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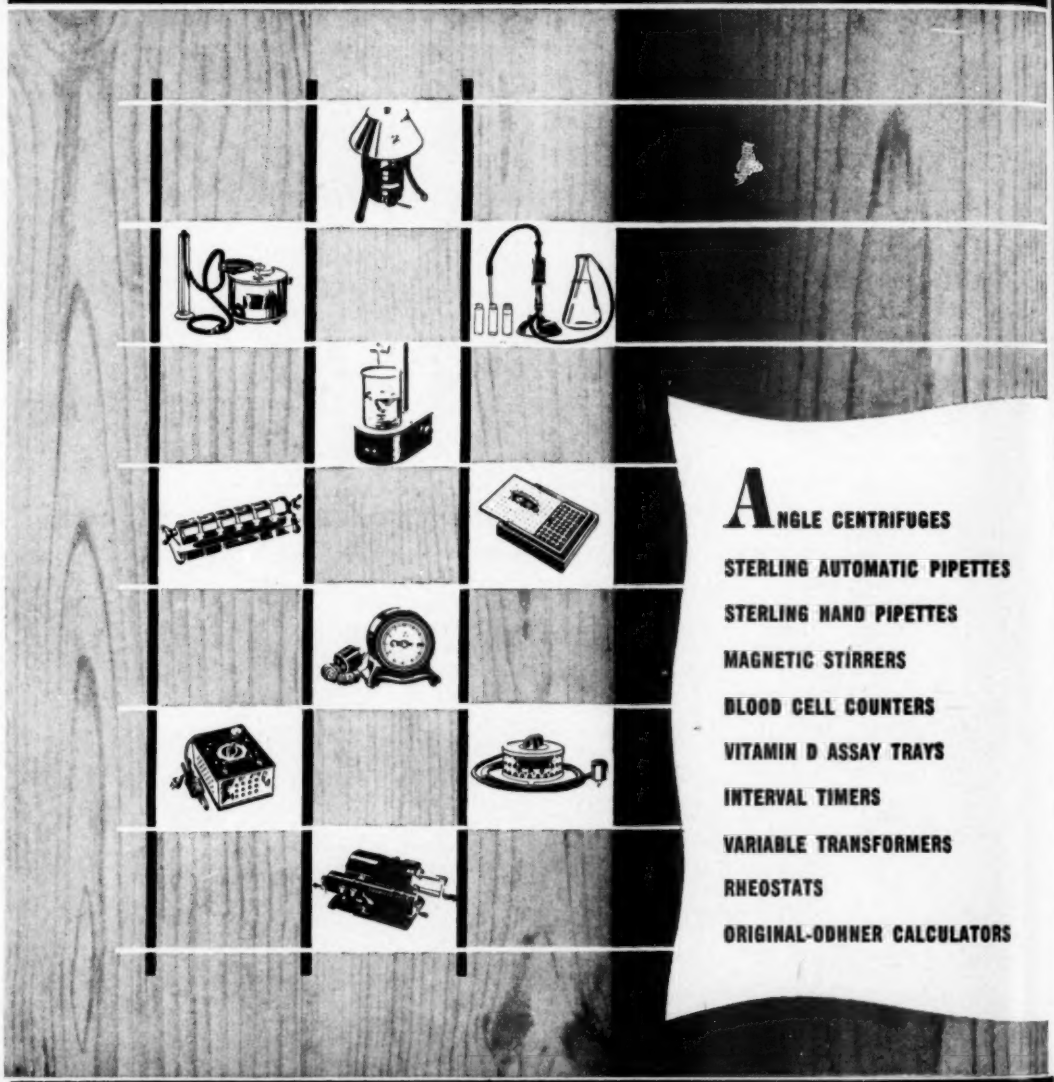

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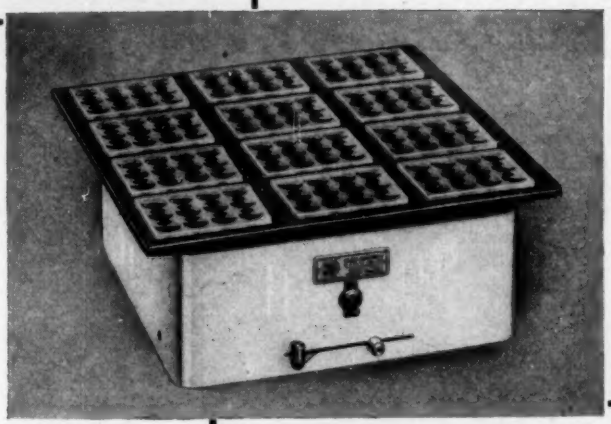

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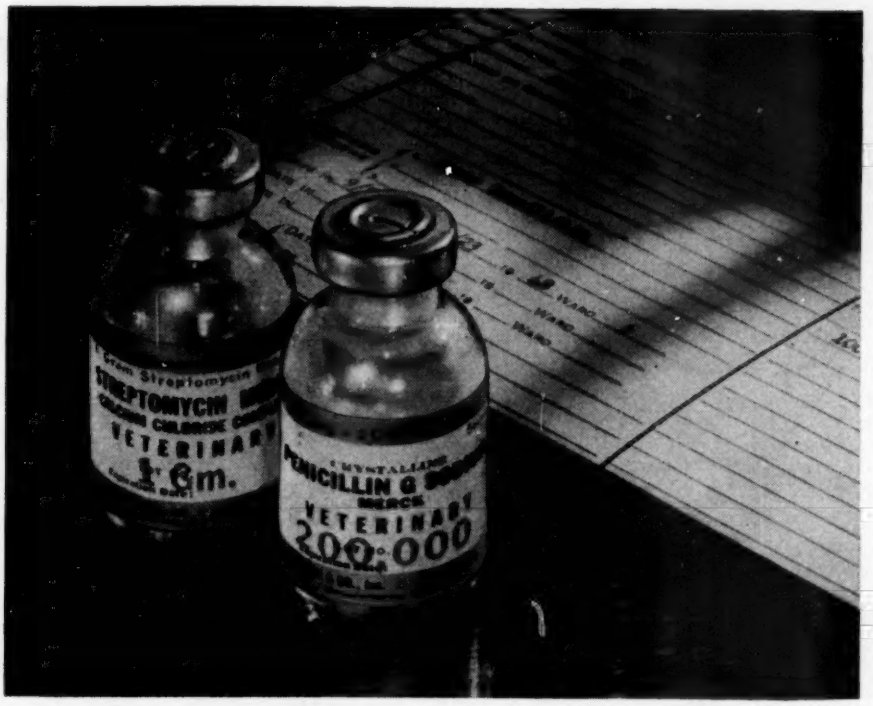

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VOL. IX OCTOBER, 1948 No. 33

Equine Sarcoid, a Cutaneous Neoplasm

CARL OLSON, JR., D.V.M., PH.D.

Lincoln, Nebraska

EQUINE sarcoid is a cutaneous tumor, usually multiple, which tends to recur following removal. The term was first applied by Jackson[5] who has given an excellent description of the disease as encountered in South Africa. There are numerous reports of tumors involving the skin of horses and mules; nearly all have considered such tumors as individual cases. The disease described by Jackson appears to be a distinct entity. Jackson[5] and Montpellier, Badens, and Dieuzeide[9] brought out evidence to indicate spread of the tumor from one site to another by such mechanical means as rubbing the head on the side or breast, and perhaps by harnesses. They also suggested that a cell-free agent might be found responsible for the tumor by analogy with buccal papillomatosis of dogs and skin papillomatosis (warts) of cattle.

Clinical and pathologic observations have been made on 2 cases of equine sarcoid,* in one of which a successful autotransplantation was done. These observations constitute the basis for this report.

CASE 1

History.—The animal was a gelding, property of the U. S. Army with brand 26F2. In January, 1938, when the horse was 10 years old, a tumor was removed from the buccal region of the right cheek. At the same time, a mass on the skin on the middle third of the right lateral surface of the abdomen was removed. Specimens from these tumors were submitted to the Army Veterinary School, Washington, D. C., for examination, and the diagnosis of neurosarcoma was made. The surgeon commented that the tumor was apt to recur. In the fall of 1940, a recurrence began to develop in the right cheek. By May, 1941, this had reached such a size that the animal could no longer be bridled without marked trauma to the mass (fig. 2). A small papillomatous tumor began to develop on the skin of the right breast in the spring of 1941. The animal remained in good flesh, and there were no symptoms suggestive of internal metastases. The animal was condemned, and permission for necropsy examination was granted.

Physical Examination.—On May 26, 1941, a firm growth was present in the subcutis of the middle portion of the right mandibular region. The mass was approximately 18 cm. long by 17 cm. broad and 7 cm. thick. It was slightly movable, adhered to the skin, and had a somewhat uneven surface due to rounded, fluctuating nodules situated deep in the substance of the tumor. There were several small fistulous tracts from which pus was draining. The skin over the mass was stretched taut. The tumor seemed to interfere but little with movement of the jaw or lips. Two small papillomatous tumors were present in the skin of the right breast. The larger measured approximately 45 mm. by 60 mm. by 15 mm. and was attached by a fairly broad base. The smaller mass measured approximately 10 mm. in diameter and 7 mm. thick with a 5 mm. base. Both small papillomas were freely movable with the adjacent skin, rough, free of hair, and encrusted with dark scaly material. The animal was in good flesh and without nervous disturbance. The skin of the body and legs was tested for areas of anesthesia and hyperesthesia with no detectable abnormality. The operative site on the right abdominal wall was marked by a healed scar.

Necropsy.—The horse was killed May 27, 1941, and necropsy examination made. Cross section of the tumor on the cheek revealed a

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*Acknowledgment is due the Veterinary Corps and Medical Department of the U. S. Army for cooperation in making these cases and records available for study. The animal of case 1 came under personal observation in May, 1941. The animal of case 2 was personally observed from April, 1944, to May, 1945. Prior to these dates, clinical and surgical work was done by other personnel of the Veterinary Corps.

tough, fibrous tissue with strands running in various directions. There were a few small encapsulated, nodular areas of gelatinous tissue and numerous abscess cavities either filled with pus or draining to the exterior. The skin was thick in some areas. There appeared to be some infiltration of the buccinator muscle but, in general, the tumor was confined to the subcutis. The facial nerve entered the mass, but its course could not be traced. Functional integrity of the motor tracts of the facial nerve was demonstrated by obtaining a muscular twitch of the upper and lower lips upon mechanical stimulation of the nerve proximal to the tumor. Both smaller tumors of the breast were confined entirely to the skin and subcutis, and no connection with cutaneous nerves could be established. On cross section, they were found to consist of dense fibrous tissue with papillomatous-like proliferation of the skin.

The right mandibular lymph nodes were enlarged and firm in consistency. No abscesses were evident. The visceral organs were essentially normal. A search was made for tumor nodules in various nerves. The right facial nerve was examined in its subcutaneous course as well as its point of origin from the brain stem. Both brachial plexuses and immediate radicals, and the intercostal and mesenteric nerves were examined. The spinal cord was dissected from the level of the first lumbar vertebra to the terminal portion of the cauda equina. No gross pathology of these nerve paths was found.

Histopathology.—The tumor of the cheek was composed principally of fibroblasts, of variable density in different parts of the tumor. In some areas, there was relatively little intercellular material. In other areas, the cells were separated by much collagen and in some places by mucinous material. Scattered throughout the tumor were the cells of a chronic polyblastic inflammation. Sections of the tissue removed in January, 1938, were made available by the Army Medical Museum. The histology was similar to that seen in tumor tissue obtained at necropsy.

The small tumors from the breast region were composed of a papillomatous-like arrangement of the epidermis and a sarcoid, fibroblastic tissue in the corium (fig. 1). The fibroblastic tissue extended to the epidermis which was drawn into deep folds and covered by a thick layer of keratinizing epithelium. In some sections, microfilarias were noted in the tunica propria. These were considered as an incidental finding.

Mitotic figures were extremely rare among the neoplastic fibroblasts. Their anaplastic character was revealed to a greater extent by irregular growth without definite pattern or purpose.

The right mandibular lymph node was hyperplastic. No gross or microscopic pathology was noted in sections of the spinal cord or peripheral nerves.

CASE 2

History.—A mule, foaled in 1936, was purchased by the Army Oct. 25, 1940, as a gelding. This animal carried brand 6K33 and was at Front Royal Quartermaster Depot, Va., from October, 1940 to April, 1943, when it was moved to Fort Dix, N. J. It was used about the post for light driving and hauling. Particular attention was given this animal from October, 1944 to May, 1945. Subsequent to May, 1945, a change of military station prevented personal observation of the case; however, through the interest and coöperation of the station veterinarian, data were kept on the course of the disease and necropsy of the animal.

The general condition of the mule was always good. It was used for work whenever the condition of the tumors near the base of the ear would permit fitting a bridle. It was killed October 8, 1945. During the period this animal was owned by the Army, five tumors developed spontaneously, and two were the result of autotransplantation.

A nodular tumor mass 8 cm. by 10 cm. and surrounding, smaller, satellite nodules on the right lateral surface of the anterior third of the neck near the base of the ear, were removed January 26, 1943. These were reported as neurofibromas upon histologic examination by the Army Medical Museum, Washington, D. C. The tumor recurred, attaining the size of a tennis ball, and was again removed on April 22, 1944. By September 16, 1944, the tumor again grew to such a size that it was surgically removed. A third recurrence developed at the dorsal aspect of the old scar and measured 5 mm. by 7 mm. on April 18, 1945 (fig. 9). Growth continued slowly, and the tumor was still present at necropsy.

A 5 cm. ovoid tumor nodule was removed from the internal surface of the right ear January 26, 1943. This was also diagnosed as a neurofibroma by the Army Medical Museum. On September 16, 1944, a recurrence of the tumor and about one-third of the ear were removed. By April 18, 1945, the growth was again well established along the edge of the ear (fig. 9). This was present at necropsy as a cauliflower-like mass occluding the external auditory meatus and covering the entire internal surface of the remainder of the ear.

A third growth was first noted September 16, 1944, on the left side (fig. 3). There were two large, firm, distinct nodules. The growth was removed October 12, 1944; it measured

10 cm. by 12 cm. (fig. 4 and 5). The operated wound healed readily and the tumor did not recur. A portion of the surgically removed tumor was used for transplantation experiments.

The fourth tumor was first observed October 12, 1944, on the left side about 14 cm. dorsal to tumor 3. It was then 1 cm. in diameter. By April 18, 1945, it had reached a size of approximately 2.5 cm. by 3 cm. and was movable with the tightly adherent adjacent skin. The entire mass with surrounding skin was removed and used for transplantation experiments. Six weeks later, this tumor had recurred and was represented by a discoid lesion of firm pink tissue with a dry surface, free of hair, and approximately 2.5 cm. in diameter. At the time of necropsy, the tumor was still present though a dark grey color.

On April 18, 1945, a small firm nodule, 8 mm. in diameter was found in the middle of the back region. It was about the same size six weeks later, though no comment was made as to its presence or absence in the necropsy data.

Necropsy.—The mule was killed October 8, 1945. The condition of the various skin tumors was as described. No other gross pathology was found at necropsy except a chronic, granulomatous reaction at the root of the mesentery, in which were encapsulated remains of nematode parasites. The brain and brain stem showed no gross or microscopic abnormality.

Histopathology. — The fundamental fibroblastic character of the skin tumors was similar to that seen in case 1. Histologic sections of tumors 1 and 2 at the time of their first surgical removal were made available for study by the Army Medical Museum. Their histology was similar to that of tissues obtained from subsequent operations. With the exception of tumor 5, each of the spontaneous tumors was sectioned for study. Those that recurred were examined after such a recurrence. There were some minor variations in density of the tumors due to the secondary effect of local edema. Variations were noted in different parts of the same tumor. For example, in tumor 3, the growth was without pattern immediately below the epidermis (fig. 6), whereas, more deeply, it tended to form whorls about degenerated muscle bundles (fig. 7). Tumor growth was not always in immediate proximity to the epidermis for in some parts of tumor 4 a considerable amount of normal corium separated the epidermis and tumor.

TRANSMISSION ATTEMPTS

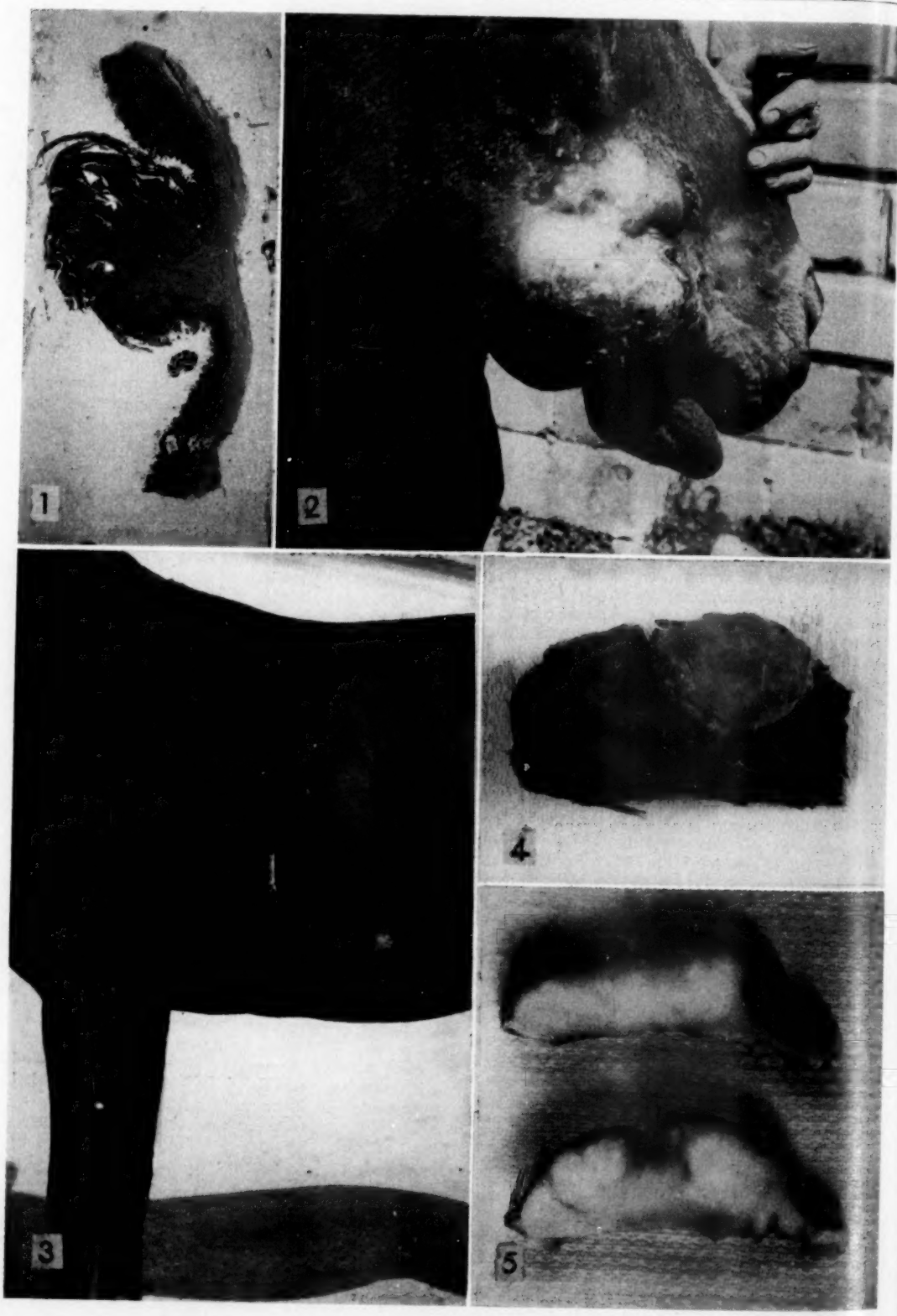
EXPERIMENT 1

Preparations and Methods of Inoculation.—On October 12, 1944, material from both the nodular masses of tumor 3 (fig. 3) of mule 6K33 was used in preparation of inoculums. There was some difficulty in making a satisfactory suspension of the tough, fibrous tissue. First the tissue was minced as finely as possible with scissors. One portion of minced tissue was put in sterile glycerine buffered at a pH of 7.0. Another portion of minced tissue was placed in a tube for transportation to the laboratory. Approximately 1 cc. of minced tissue and 30 cc. of sterile Ringer's solution were ground together to form a suspension. Part of this suspension was filtered through a Swinney type Seitz asbestos filter. The efficacy of filtration was tested by adding a suspension of *Serratia marescens* just prior to filtration. Filtrate seeded on agar culture tubes was sterile, thus demonstrating retention of bacteria by the filter. Approximately 1 cc. of ground suspension and filtrate was placed in each of two tubes. These were immersed in boiling water for five minutes.

The hair over an area 10 by 10 inches was closely clipped on both sides of the neck of mule 6K33. The lower half of each area was shaved. The entire area was washed and scrubbed with soap and water, followed by a wash with acetone to remove oil from the skin. The sites for injection of material were approximately 2 in. apart, and each was carefully marked by encircling with a wax pencil. The right side of the neck was inoculated with fresh material, and boiled material was used on the left side of the neck. Inoculations were made subcutaneously, intradermally, by scarification, and on intact skin with about 0.05 cc. at each site. Each type of inoculation was made in duplicate; thus there were 16 exposure sites on each side of the neck. Inoculations were commenced within an hour after removal of the tumor and completed in about forty-five minutes.

Scarification was accomplished by several light scratches with the point of the needle. These were sufficiently deep to cause slight oozing of clear or only slightly bloody fluid. Inoculum was placed on the area without subsequent rubbing. Intact skin was inoculated by simply placing the material on the skin. Both the scarified and intact skin inoculation sites were protected by surrounding them with sterile rubber washers. These were covered with sterile, paraffined gauze sponges, sealed over and held in place by adhesive tape. These protective coverings were used to delay drying of the inoculum. The coverings had loosened, and the sites were unprotected six hours later.

Minced tissue, a glycerinated specimen, and minced tumor that had been dried *in vacuo* over sulfuric acid were placed in a refrigerator. These were used one week later to inoculate a horse.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On Oct. 19, 1944, a 14-year-old mare (167E), condemned because of "heaves" was made available as a test animal. An area on the right side of the neck 9 in. by 8 in. was closely clipped and the lower half shaved. The area was scrubbed with soap, dried, and then washed with acetone. Twenty inoculation sites were marked on the prepared area. Inoculations were made subcutaneously, intradermally, on scarified skin, and on intact skin in 0.05-cc. amounts. The following inoculums were used: minced tissue suspension; filtrate of tissue suspension; a 1 : 10 suspension of dried tumor in Ringer's solution; 0.5 cc. ground, glycerinated tissue suspended in 2 cc. Ringer's solution; and Ringer's solution alone as a control. Scarified inoculations were done by making several scratches with a needle until there was oozing of clear or slightly bloody fluid; inoculum was then placed on the area and rubbed with the rounded end of a sterile glass rod. These and the intact skin inoculations were covered with sterile paraffined gauze pads held by adhesive tape. The protective coverings had loosened after from six to eight hours.

Results.—No evidence of growth was noted at any of the inoculation sites on mule 6K33 until six months later, when two small nodules, each 1 cm. in diameter, were apparent on the right side of the neck (fig. 9). They were at the sites of inoculation with tumor suspension on scarified skin and were designated tumors 6 and 7. April 18, 1945, one (tumor 6) was removed. Its histologic structure was the same as in the spontaneous tumor (fig. 8). No recurrence followed its excision. Tumor 7 continued to grow and was present at necropsy of the animal, a year after inoculation. All other inoculation sites remained negative.

At no time did tumor formation occur at the sites of inoculation on horse 167E. This animal was under observation for thirty-six weeks after inoculation. It was killed June 28, 1945. Necropsy revealed chronic emphysema of the lungs and no evidence of internal change due to the inoculations with tumor material.

EXPERIMENT 2

Preparation and Methods of Inoculation.—April 18, 1945, tumor 4 of mule 6K33 was removed. A slice of tissue from the middle of the tumor was minced with sterile physiologic salt solution, then ground to a finer suspension. A closely clipped and shaved area on the left side of the neck of mule 6K33 was inoculated with the tumor suspension. Approximately 0.2-cc. doses were used . Two inoculations were made intradermally and two on areas scarified with a needle until hemorrhagic lymph oozed out. The inoculum was rubbed into the latter sites with the sterile end of a rounded glass rod. Then the area was covered with sterile paraffined gauze and adhesive tape.

About six to seven hours later the same material was inoculated into the skin of two horses (14 and 22 years old) and two mules (21 and 22 years old): The methods and procedure were the same as previously described. One of the horses was 167E, used in experiment 1.

Results.—No tumor developed at any of the inoculation sites in this experiment. Horse 167E was killed seventy-one days later. Skin in the vicinity of the inoculation was apparently normal. The sites of inoculations could not be located with sufficient precision to warrant histologic section. Mule 6K33 was killed 173 days later, and apparently there was no reaction, for no comment was made in the available necropsy data. The sites were specifically examined six weeks after inoculation and found normal. The remaining horse and 2 mules showed no reaction ten months after inoculation and were considered negative.

DISCUSSION

Both case 1 and case 2 fit the description given by Jackson[5] for equine sarcoid. The series of 44 cases studied by Jackson provides more information on the common sites of these tumors which were the lower portions of the legs, eyelids, lips, other parts of the skin of the head, sheath of the penis, and inner aspect of the thigh. Montpellier and his collaborators[9, 10] have taken cognizance of the disease in Algeria, calling it a Schwann tumor. The similarity of findings and deductions in Algeria and South

PLATE I

Fig. 1—Histologic appearance of the breast tumor, case 1. Note the resemblance to a benign, true papilloma. X 3.75.

Fig. 2—Case 1, illustrating the large, irregular growth. Openings of fistulous tracts draining abscesses are apparent.

Fig. 3—Tumor 3 of case 2 prior to its removal. Successful autotransplant was obtained from this growth.

Fig. 4—Exterior surface of tumor 3, case 2. Lack of skin covering over the two large nodules of this tumor is evident.

Fig. 5—Cross sections of tumor 3, case 2, showing tumor tissue between the two large nodular masses.

Africa are remarkable, since each group of workers was evidently unaware of work of the other. Montpellier, *et al.*[9] describe 6 cases in detail (4 in castrated males and 2 in female mules) and comment on the condition having been noted frequently in mules and horses. Natural autoinoculation of the tumor by contact was pointed out by them in the cases with growths on the skin of the sheath or mammae and adjacent thigh region. In one case, the tumor appeared at the site of a castration wound.[3]

Equine sarcoid has no doubt existed in this country under various names without being recognized as an entity. For example, the surgeon who operated on case 1 gave a prognosis of probable recurrence. The original diagnosis of neuroma in both cases 1 and 2 is logical from the histologic appearance of well-developed tumor nodules. Runnels and Benbrook[14] reviewed 90 cases of malignant sarcoma from horses and mules diagnosed at Iowa State College. Fibrosarcoma was diagnosed in 81 instances, myxofibrosarcoma in 5, and myxosarcoma in 1. Three other tumors contained osteogenic elements. In 76 instances, the site was where equine sarcoid might be expected. Recurrence was recorded in 11 cases though the authors suggested there may have been more as the records were considered incomplete. No evidence of internal metastases was found. It is not inferred that these malignant sarcomas were equine sarcoid, but the description of some suggests this possibility. Among the 64 benign tumors were 27 fibromas, most commonly located on the leg, head, shoulder, pectoral region, and nasal sinus.[14] Frank,[3] figure 66, illustrated a case of multiple nodular fibrosarcoma in a horse. This was diagnosed by histologic section, though the sections were no longer available for examination.[4] Frank[4] has observed, in mules, fibrosarcomas on the skin of the head, neck, or shoulders which have invariably returned either at the point of removal or some other part of the skin. According to Feldman,[2] fibromas and fibrosarcomas of the subcutis are not uncommon in the horse and mule. He states that the condition of multiple primary fibroblastic tumors of the integument, designated as cutaneous neurofibromatosis, occurs most frequently in horses. This leads to a consideration of multiple neurofibromatosis or von Recklinghausen's disease of man. The scope of this disease in human pathology is still not known, though it does include several varieties of neoplastic changes in the connective tissue of the nervous system and seems to have an hereditary basis.[11] Some good examples of disease which fulfill the requirements for multiple neurofibromatosis do occur in domestic animals, particularly cattle.[12, 6] In this condition, neoplastic-like nodules are found on the major nerves themselves. The rather uniform failure to find these lesions along the major nerves in equine sarcoid would serve to differentiate between the two diseases.

The term equine sarcoid, first proposed by Jackson,[5] seems fitting for the disease. It emphasizes the neoplastic character of the growth and implies the potentiality for spread. The term sarcoid in itself suggests a sarcomatous lesion. While the term might cause some to associate the equine disease with the entirely different disease of Boeck's sarcoid in man, an understanding of the pathology of these two diseases will lead to clear distinction. Equine sarcoid cannot be considered as a benign fibroma. Neither can it be thought of as a malignant fibrosarcoma. Some term to indicate it as an entity is required. The peculiar neoplastic predisposition of connective tissue of the equine corium characterizes the disease. Whether the mesothelium of the tumor is derived from corium proper, from Schwann sheaths of cutaneous nerves, or elsewhere is not known, but mesothelium in parts of the body other than skin does not seem to become affected.

Jackson[5] reconstructed the pathogenesis of equine sarcoid from his series of cases. First there is proliferation of the epidermis and connective tissue of the cutis in which the lesion can be recognized as a "papilloma" (fig. 1). Soon the fibroblastic growth develops more rapidly; the epidermis becomes thin, breaks, and an ulcer forms. Lateral growth of the tumor in-

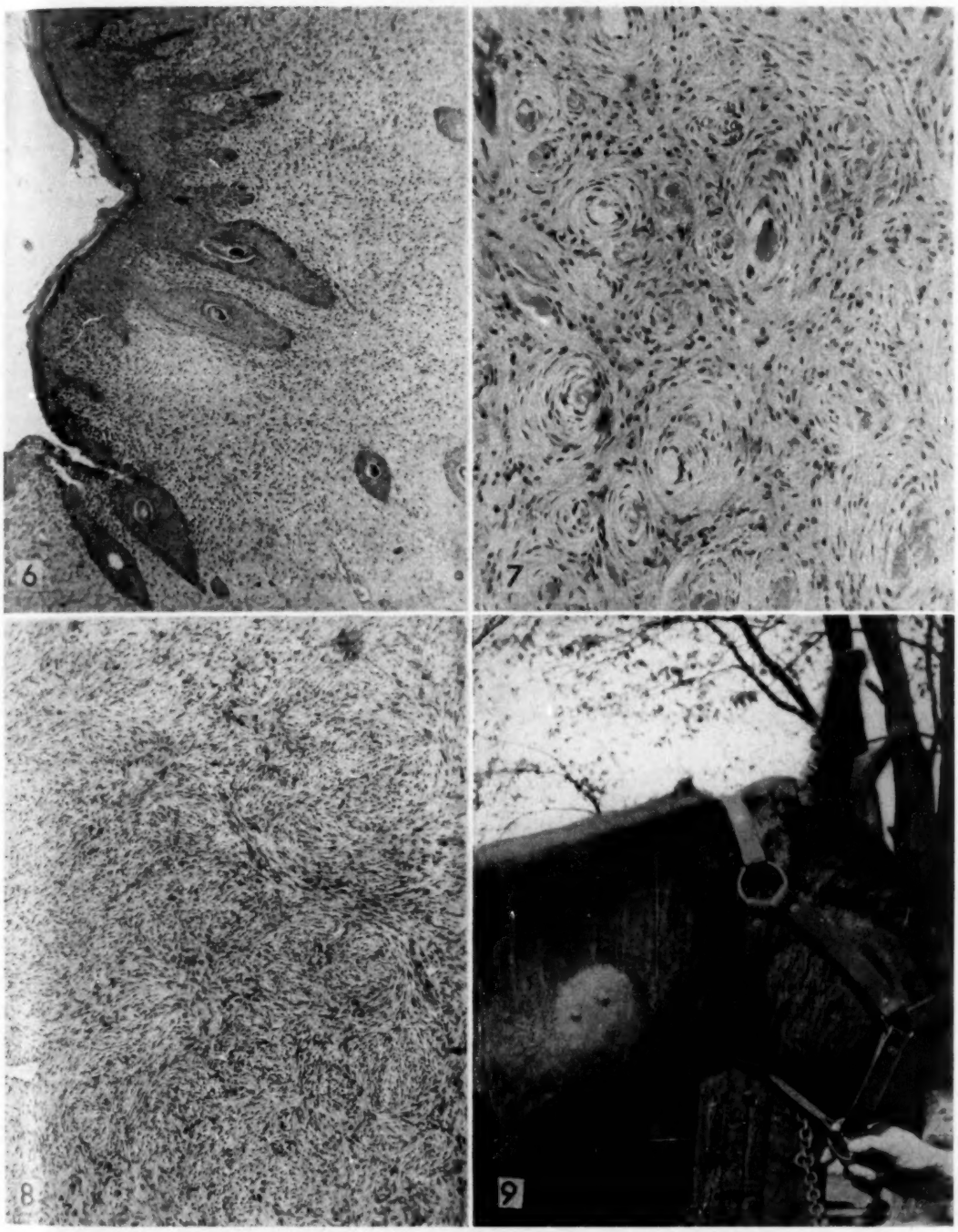

PLATE 2

Fig. 6—Photomicrograph of tumor 3, case 2, showing complete replacement of corium by sarcoid tissue. X 60.

Fig. 7—A deeper portion of the same tumor illustrated in figure 6 showing whorled arrangement of sarcoid cells. X 125.

Fig. 8—Photomicrograph of tumor 6, case 2, which illustrates the neoplastic appearance of the sarcoid tissue resembling a fibrosarcoma. X 90.

Fig. 9—Tumors 6 and 7, case 2. These growths occurred where a suspension of sarcoid tissue had been placed on scarified skin six months previously. Recurrence of growth of tumors 1 and 2 may be noted near the base and along the edge of the right ear.

creases its size. The ulceration leads to bacterial infection and subsequent inflammatory change. The histologic appearance of equine sarcoid in the "papilloma" stage is identical with that of a benign, true papilloma.

Experiment 1 demonstrated successful autotransplantation of equine sarcoid by means of a tissue suspension inoculated on scarified skin. The possibility that the tumors might be due to localization of circulating emboli of tumor cells or a substance capable of producing tumor in an area of low resistance was controlled by using similar, though boiled, material in other sites. The boiled material was inactive. Furthermore, the positive as well as negative results were obtained in duplicate. No satisfactory explanation can be offered for the failure to obtain the same result in experiment 2. Attempts to secure growth of implants in other horses and mules were not successful, though the experiments were open to criticism. In experiment 1, there was an interval of seven days between successful autotransplantation in mule 6K33 and inoculation of the horse. This difficulty was overcome in experiment 2. However, in this trial, the material was ineffective on autotransplantation. These attempts should be repeated. Cadeac[1] describes two experiments in which a horny papilloma was reproduced on the skin of healthy horses. The donor was an adult animal with large plaques of wart-like growth on the breast. The gross illustration of this case is suggestive of equine sarcoid. Tumor material was ground and inoculated on scarified skin. First evidence of the inoculated tumor was fresh granulation at twenty days in one case and at about thirty days in the other case. Cadeac mentions having observed recurrence at the site of needle and suture puncture during operation on other cases of this type, suggesting autoinoculation. He also mentions contact infection as a method of spread in an individual case. Montpellier, *et al.*[10] have reported the successful subcutaneous graft of what they term a "Schwann tumor" in a mule. This type of tumor was no doubt similar to Jackson's equine sarcoid. In their experiment, a pea-sized piece of a tumor from the skin of the ventral region was placed under the skin of the neck of the same mule. This appeared to become absorbed in a month. Two years later a 10 cm. elongate growth with histologic structure similar to the parent tumor was found at the site of transplantation.

The autotransplantability of equine sarcoid is not necessarily a unique characteristic. Ribbert[13] obtained successful autotransplants with a hard fibroma primary on the abdomen of a dog. The autotransplants developed to about 4 cm. in four months. Implants to another dog failed to grow. Mann[8] obtained successful autotransplants with a fibroma in a cat which did not grow in 25 other cats to which transplants were made. The fibromas of the donor cat did not show the tendency to become multiple.

Experiment 1, in which equine sarcoid was transplanted to scarified skin, provides a controlled demonstration of how these growths might be spread to different areas of the same animal, substantiating the observations of Jackson[5], Montpellier, *et al.*,[9, 10] and Cadeac.[1] The failure to obtain growth when the same material was inoculated intradermally or subcutaneously is difficult to explain. Perhaps a regenerating epithelial surface was required as a seed bed for initial growth of the sarcoid. Intact skin appeared to be invulnerable.

SUMMARY

Two cases of equine sarcoid are reported which fit the description of the disease as encountered in Africa. Features of equine sarcoid indicating it as a disease entity are discussed.

Successful autotransplantation of the tumor to scarified skin was obtained in 1 case.

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Statement of Ownership and Management Required by the Acts of Congress of August 24, 1912, as amended by the Acts of March 3, 1933, and July 2, 1946, for the American Journal of Veterinary Research. Published Quarterly at Chicago, Ill., as of Oct. 1, 1948: "State of Illinois, County of Cook, before me, a notary public, in and for the state and county aforesaid, personally appeared Dr. J. G. Hardenbergh, who, having been duly sworn according to law, deposes and says that he is the managing editor of the American Journal of Veterinary Research and that the following is to the best of his knowledge and belief a true statement of the ownership and management of the aforesaid publication for the date shown in the above caption, required by the Act of August 24, 1912, as amended by the Act of March 3, 1933, embodied in section 537, Postal Laws and Regulations, to-wit:

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Helen S. Bayless, Notary Public (commission expires October, 1949)."

Studies on the Pathogenicity of Brucella Suis for Cattle. I

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THE QUESTION of how *Brucella suis* gains access to the mammary glands of cattle has been of concern to research workers and public health officials for many years. Whether swine infected with *Br. suis* can transmit the disease to cattle and, if so, under what conditions are important considerations from a public health standpoint. Dr. C. F. Jordan[1] states, "From time to time a raw milk supply becomes contaminated with *Brucella suis* brought about by hogs sharing the same lot with dairy cows. When infection is transmitted from infected hogs to the udder of one or more dairy cows, multiple cases of brucellosis are certain to result."

The initial work was set up to determine the potential danger of infected swine sharing the same lot with dairy cows. Later work was done under highly experimental conditions to determine, if possible, a route by which *Br. suis* would gain entrance to, and establish infection in, the bovine udder with concurrent elimination of viable *Br. suis* organisms.

REVIEW OF LITERATURE

Huddleson[2] states that there is insufficient evidence to indicate exactly what rôle the infected hog plays in the transmission of the disease to cattle. On the basis of work carried out at the Central Brucella Laboratory, he concludes that *Br. suis* is involved in infection of cattle in many of the southern states.

Cotton,[3] on the basis of research carried out at Beltsville, Md., thought that in cattle and swine Brucella infections were probably not intercommunicable to any great extent by natural means though they are through inoculation. He[4] also thought that any danger of the disease being transferred by natural exposure from swine to cattle causing any more than a transient infection was remote.

Cotton and Buck[5] reported that the porcine strain could cause infection of cattle by intravenous injection of a heavy suspension. They thought that the swine strain is only mildly infectious for cattle by natural means but that the organism may gain access to the udder and, at times, the uterus, under some unusual forms of exposure, and occasionally even cause abortion.

Cotton, Buck, and Smith[6] found that pregnant cattle can sometimes be infected and even caused to abort by very severe exposure to *Br. suis* through the conjunctiva or through the conjunctiva and digestive tract. Of 8 cows thus exposed, 3 became infected. They concluded that cattle give evidence of possessing considerable resistance to infection with the porcine type and seldom contract the disease from swine as a result of natural exposure. Harms[7] concluded that cattle sometimes may become naturallly infected with the porcine strain of *Brucella abortus*.

Howarth and Hayes[8] reported experimental results in which a Holstein-Friesian cow, reacting negatively to the blood serum test and originating from a negative herd, reacted positively three months after being placed in a small enclosure with 12 reacting sows, 8 of which farrowed during the course of the experiment. Starr[9] applied viable cultures of *Br. suis* to the scarified skin of a 5-weeks-old calf and to the eye of another calf of the same age. He reported the appearance of low titer agglutinins which disappeared after a short time. Elder[10] failed to demonstrate *Br. suis* infection in 8 cows kept in a small lot with infected swine. He found that some of the animals developed low agglutination titers which were transient in nature and, although four abortions occurred, bacteriologic examinations for either *Br. suis* or *Br. abortus* were negative.

Hayes[11] maintained that the frequent isolation of *Br. suis* from the udders of cows emphasized the potential danger of the swine infection to cattle and man. Hasseltine[12] reported that both *Br. abortus* and *Br. suis* were isolated from cows' milk. Plastridge and McAlpine[13] reported the isolation of 46 cultures of *Br. suis* out of 129 cases of undulant fever in human beings. Beattie and Rice[14] reported on a milk-borne epidemic of undulant fever involving 30 cases. Of the 30

TABLE I.—Results of Exposure of Cows to Brucella Suis

Abridgement of a thesis by Dr. Washko presented to the faculty of the graduate school, Purdue University, in partial fulfillment of the requirement for the degree of master of science, February, 1948. Journal paper 345, Purdue University Agricultural Experiment Station, Lafayette.

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TABLE I—Results of Exposure of Cows to Brucella Suis

Cow	Stage of preg. at time of exp.	Agglutination response in months post exposure									Parturition		
		1	2	3	4	5	11	16	19	21	24	30		Cult. results‡	Co os. titer
						Exposed to *Br. suis* infected swine									
A	6 mo.	II---	I----	-----	I----	+*----	-----		-----			-----	N.	Neg.	
B	Open	II---	++II-	+I---	I----	I----	Removed from the experiment								
C	4 mo.	II---	II---	II---	I----	I----							N.	Neg.	+I---
D	6 mo.	I----	+II--	I----	II---								Dyst.†	Neg.	
						Exposed to aborted swine fetus *per os*									
E	7 mo.	III--	I----	I----	I----	-----	-----	-----	-----		-----		N.	Plac. Pos.	+++++
F	Open	++II-	++II-	II---	II---	III--	-----	II---			-----		N. N.	Neg. Neg.	I---- -----
G	4 mo.	++III	+III-				I----	I----			-----		N.	Neg.	+++II
H	Open	+++++	+++II				-----	+II--			-----		N.	Neg.	-----
													N.	Neg.	++II-
I	2 mo.	+-I-	-II---				-----	-----					N.	Neg.	

+ = complete agglutination; I = incomplete agglutination; Neg. = negative; Pos. = positive; N. = normal, indicates calving was full term with negative bacteriologic results for *Brucella suis*. *Fed known infected swine fetuses. †Dystocia; retained membranes. ‡At parturition.

cases, 27 obtained their milk from the same raw milk supply. *Brucella suis* was reported as being isolated from the blood stream of 6 patients and from the milk of one of the cows in the herd.

Borts, Harris, Joynt, and Jennings[15] traced an epidemic of undulant fever to *Br. suis* in a raw milk supply. Fitch and Bishop[16] stated that the organism found in milk is usually *Br. abortus*, but that it has been demonstrated that *Br. suis* may invade cattle and cause infection. Hardy, and associates,[17] in Iowa, reported 45 of 61 cases of undulant fever as due to *Br. suis*.

Runnels and Huddleson[18] reported on inflammatory foci in the mammary tissues of cows infected with *Br. abortus*, although they observed no Brucella in histopathologic sections. Sholl and Torrey[19] concluded that *Br. abortus* produces definite changes in the udder which may be quite extensive and may lower the efficiency of the organ considerably. Hallman, Sholl, and Delez[20] found that *Bacterium abortus*-infected udders showed a subacute or chronic interstitial mastitis in the majority of cases. They thought that these tissue alterations were responsible for the increased leucocyte content of milk from *B. abortus*-infected udders.

EXPERIMENTAL

In the first series of experiments, 9 cows, 6 pregnant and 3 nonpregnant, were exposed to *Br. suis* as follows: 4 animals, A, B, C, and D, were exposed to *Br. suis*-infected pigs by intimate pen contact for from three to five months. Cow A was both pen-exposed to infected swine and fed aborted swine fetuses and membranes. The remaining 5 cows were exposed to the infection by feeding aborted swine fetuses and membranes known to contain *Br. suis*. In addition to such known exposures, these cattle were maintained in close proximity to infected swine.

Twenty-one to thirty months following exposure of these cattle, 6 of the original 9 were exposed through the conjunctiva to approximately 210 million *Br. abortus* organisms. Simultaneously, 4 adult cows, 4 heifers, and 2 bulls were exposed similarly and used as control animals.

Serum agglutination tests were conducted on all cows at irregular intervals. The rapid agglutination test was employed with serum dilutions of 1 : 25, 1 : 50, 1 : 100, 1 : 200, and 1 : 400.

Bacteriologic examinations for both *Br. suis* and *Br. abortus* were conducted on colostrum, fetal membranes, and dead or aborted calves at parturition.

The second series of experiments was conducted on lactating dairy cows reacting negatively to the blood test and originating from

TABLE 2—Results from Exposure of Cows to Brucella Abortus

Cow	Stage of preg. at time of exp.	Agglutination response in months post exposure						Parturition			
		1	2	3	4	5	6		Cultural results	Colostral culture	Colostral titer
		Exposed to *Br. abortus* per conjunctiva, after previous exposure to *Br. suis*									
A	Sterile	I I - - -	I I - - -	- - - - -	I I - - -	- - - - -	I I - - -	N.			
E	8 mo.	+ + - - -	I I I - -	I I I - -	+ - - - -	I I - - -	I - - - -	N.	Pos.	Pos.	+ + + + I
F	Open	+ + I - -	+ + I I -	+ I I - -	+ I I - -	I I - - -	+ I I - -				
G	2 mo.	+ + + I -	+ I I I -	I I - - -	I - - - -	I I I - -	- - - - -	D.F.	Neg.	Neg.	- - - - -
H	6 mo.	I I I - -	I I I - -	+ I I I -	+ I I - -	+ I I I -	+ I I - -	N.	?	Pos.	- - - - -
I	7 mo.	+ + + I -	+ + + + I	+ + + I I	I I I - -	+ + I I I	I I - - -	N.	Pos.	Pos.	+ + + + +
		Controls, exposed only to *Br. abortus*									
J	Open	I I I I -	+ + + + +	+ + I I -	+ + + I -	+ I I - -	+ I I I -				
K	3 mo.	I I - - -	+ + + I -	- - - I I	+ + + + +	+ + + I I	+ + + + +	A.D.C.	Pos.	Pos.	+ + + + +
L	Open	+ + I I -	+ + + + +	+ + + + I	+ + + + +	+ + + + +	+ + + + +				
M	7 mo.	+ + + + -	+ + + + +	+ + + + +	+ + + I I	+ + + + I		A.L.C.	Pos.	Pos.	+ + + + +
N	Open	I I - - -	- - - - -	I - I - -	- - - - -	+ + I - -	I - - - -				
P	Open	+ + - - -	I I - - -	I I - - -	+ + + + -	I - - - -	+ + I I -	A.	?	Pos.	
Q	Open	I I I - -	+ + I - -	+ + + I I	+ + + + I	+ + + - -	I - - - -				
R	Open	- - - - -	- - - - -	I I - - -	- - - - -	I I - - -	I I I - -				
S	Male	I I - - -	+ + + I -	I I I - -	- - - - -	I - I I -	I I - - -				
T	Male	I - - - -	+ I I I -	+ I I - -	I I - - -	I - - - -	+ I I I -				

+ = complete agglutination; I = incomplete agglutination; N. = normal, indicates calving was full term.
D.F. = dead fetus; A.D.C. = aborted dead calf; A.L.C. = aborted live calf; A. = aborted.
Pos. = positive; Neg. = negative; ? = not done.

a known brucellosis-free herd. These animals were at all times maintained in complete segregation from each other, the only means of contact being through the caretakers. The 5 cows employed in conducting the experiment were exposed to virulent cultures of *Br. suis* by various routes as follows: cow 1 (4 months pregnant) was exposed to a polyvalent culture of *Br. abortus* (laboratory cultures 1414, 1415, and 1416) through the conjunctiva to the extent of approximately 5 million organisms. A sufficient period of time (1 month) was allowed to establish this infection, following which, the animal was exposed through the conjunctiva to a polyvalent culture of 3 billion *Br. suis* organisms (laboratory strains 67, 64, and 4041). Approximately one month later, the animal was re-exposed to the suspension of *Br. suis* (number not determined) by the same route, *i. e.*, through the conjunctiva. Cow II (3 months pregnant) was exposed cutaneously to a polyvalent culture of *Br. suis* (laboratory strains 64, 67, and 4041). The areas over the left front and left rear quarters were clipped free of hair, washed with soap and water, and further cleaned with alcohol. These areas were abraded and a virulent culture of *Br. suis* was applied. The procedure was repeated approximately one month later. Cow III (5½ months pregnant) was exposed by introducing a polyvalent suspension of *Br. suis* (laboratory cultures 1779, 4041, and 67) directly into the milk cisterns of the right front and right rear quarters by employing a milk tube and syringe. Approximately 50 million organisms were injected into each quarter. The left front and left rear quarters were not exposed. Cow IV (nonpregnant) was fed a recently aborted swine fetus known to contain large numbers of *Br. suis*. Cow V (4 months pregnant) was placed in a lot with 2 recently infected (*Br. suis*) pregnant gilts. All animals in this lot were fed from the same feeders and were kept in as close contact as possible in order to provide maximal natural exposure.

Milk examinations were made four times per week. Samples were drawn on Monday, Wednesday, and Friday evenings and Saturday mornings. The milk samples were allowed to stand for twelve hours, and the cream was removed and plated on standard tryptose agar (Difco) plates and incubated aërobically at 37 C. in all cases except that of cow 1 in which the samples were also incubated under 10 per cent CO_2 tension. Brucella organisms isolated were examined serologically and also by the dye plate method of Huddleson.[21, 22]

Blood examinations were made at regular intervals, and the agglutination responses and bacteriologic studies effected. For bacteriologic study, approximately 60 cc. of blood was drawn aseptically from the jugular vein and incubated at 37 C. in 100 cc. tryptose broth (Difco) for two weeks, during which two platings were made.

Bacteriologic examinations for Brucella were conducted on colostrum, fetal membranes, and dead or aborted fetuses when possible.

The third group of cows comprised 6 pregnant, adult cows originating from a brucellosis-free herd and individually reacting negatively to the blood test. The animals were allowed to mingle freely in a large pasture, and no attempts were made to effect segregation in any way. The animals employed in these trials were exposed as follows: cows 0 and 1 were exposed by injecting a polyvalent suspension of *Br. suis* directly into the milk cisterns of the right front and right rear quarters. Approximately 1 billion organisms were injected into each quarter. Cows 2 and 3 were exposed by applying repeatedly a heavy suspension of *Br. suis* (numbers not determined) in the open teat orifice of the right front and right rear quarters. Cows 4 and 5 were exposed by applying *Br. suis* to the abraded skin over the left front and left rear quarters. The exposure was repeated twice at irregular intervals.

TABLE 3—Results of Milk Examinations of 5 Cows Exposed to Brucella Suis by Various Routes

Cow No.	Route of exposure	No. of exams	No. of isolations and organism recovered			
			RF	RR	LF	LR
I	Conj.	96	3 *Br. abortus*	None	None	None
II	Cut.	63	2 *Br. suis*	2 *Br. suis*	None	None
III	Inj. into Teat	79	37 *Br. suis*	15 *Br. suis*	None	None
IV	Os	34	None	None	None	None
V	Exp. pen	68	None	None	None	None

RF = right front quarter; RR = right rear quarter.
LF = left front quarter; LR = left rear quarter.
*Cow exposed to *Br. abortus* and subsequently exposed to *Br. suis*.

TABLE 4—Results of Blood Examinations of 5 Cows Exposed by Various Routes

Cow No.	Pre-exp. titer blood culture	Route of exp.	Date of exp. and re-exp.	Agglutination titer and blood culture results							Parturition	
				6/11	7/25	8/16	8/22	9/12	10/17	12/11	1/16	Coos. culture results
I	- - - - - -	Conj.	6/ 9/47 9/10/47	- - - - - Pos.*	+PT- -	PP- - -	+PT- -	+++T-	+++PP			Ab. Pos.
II	- - - - - -	Cut.	7/14/47 8/13/47		+PT- -	PT- - -	+PT- -	PT- - -	T- - - -	T- - - -	- - - - -	N. Neg.
III	- - - - - -	Teat canal	8/ 4/47			+++++	+++PP	+++++	+++++	+++T-	- -PT-	N. Neg.
IV	- - - - - -	Os	8/13/47				T- - - -	+- - - -	- - - - -			
V	- - - - - -	Pen	8/13/47				PT- - -	T- - - -	T- - - -	PT- - -	T- - - -	N. Neg.

+ = complete agglutination; P = partial agglutination; T = trace of agglutination; Neg. = negative; N. = normal.
*Pos. indicates *Brucella abortus* was recovered.

Milk and blood cultural examinations were made and the agglutination responses studied as in the previous group. Postparturitional bacteriologic examinations were made on colostrum and fetal membranes when available.

RESULTS

The results of the intimate pen exposure of 4 Brucella-free cows to *Br. suis*-infected swine are shown in the first part of table 1. Cows, B, C, and D showed a slight transient agglutination response, but *Br. suis* was not established in any of these 4 cows, as evidenced by bacteriologic examination of fetal membranes and colostrum at parturition. Bacteriologic examinations at autopsy of the tissues of cows C and D were negative for this organism.

The results of feeding known *Br. suis*-infected swine fetuses and membranes to 5 cows are shown in the second part of table 1. All 5 cows developed a slight agglutination response but, here again, the response proved to be transient in nature. Calvings were normal in all instances, and *Br. suis* was not recovered except in the case of cow E from which it was recovered from the placenta, despite a negative agglutination titer at the time of parturition. Colostral agglutination titers were positive in cows E, G, and H. The serum agglutination titers of all 5 animals decreased rapidly following the postexposure rise and were insignificant by the fourth month after exposure.

The results of the exposure to *Br. abortus* of 6 cows previously exposed to *Br. suis* are shown in the first part of table 2. Moderate rises in agglutination responses were demonstrated in 5 of the 6 cows. Only 4 of the 6 cows were pregnant at the time of exposure to *Br. abortus;* 3 completed full term pregnancies, and the organism was isolated from the colostrum of all 3 cows and from the fetal membranes of 2. The fourth animal gave birth to a dead fetus and cultural results for Brucella were negative.

The results of exposing 4 adult cows, 4 heifers, and 2 young bulls (all Brucella-free) to the same inoculum of *Br. abortus* through the conjunctiva are shown in the

second part of table 2. This group was used to check on the virulence of the cultures used on the previously *Br. suis*-exposed animals. The 4 adult cows (J, K, L, and M) showed a marked agglutination response, and the 2 pregnant animals aborted. *Brucella abortus* was isolated from the aborted fetuses, membranes, and colostrum. The 4 heifers (N, P, Q, and R) showed variable low titered, agglutination responses following exposure. Heifer P aborted and *Br. abortus* was recovered from the colostrum of this animal. Fetal membranes and the aborted fetus were not obtained for bacteriologic study. The remaining 3 heifers were not pregnant. Both males showed a low titer, variable, agglutination response following exposure.

The results of the milk examinations of the second group of cows are shown in table 3. From cow I, *Br. abortus* was recovered only three times in a total of 96 examinations. *Brucella suis* was not recovered from the milk of cow I. Of a total of 63 examinations of milk from cow II, *Br. suis* was recovered from the right front and right rear quarters twice. It should be mentioned that there was a very strong possibility, or almost a certainty, that the milk samples of cows II and III were interchanged on the days that *Br. suis* was recorded as being recovered from cow II. At the time of such recoveries from cow II, recoveries were not made from cow III, which was eliminating these organisms in the milk continuously during this period. Of a total of 79 milk examinations from cow III, *Br. suis* was recovered from the right front quarter 37 times and from the right rear quarter 15 times. Isolations in this case were at first continuous but, later, the elimination of the organisms assumed an intermittent character. In 34 milk examinations from cow IV and in 68 milk examinations from cow V, no recoveries of *Br. suis* were effected.

The agglutination responses, blood cultural, and calving results are shown in table 4. In only 1 case was Brucella recovered from the blood stream, and this proved to be *Br. abortus*, recovered from the blood stream of cow I eight days after exposure. Agglutination responses followed much the same pattern as found in the previous group of cows exposed to *Br. suis*, with the exception of cow III, which reacted positively at a serum dilution of 1:400 within twelve days after exposure. Unfortunately, the highest dilution at which agglutination occurred was not determined at this time. In this animal, unlike those in the previous group, the agglutination response remained significantly high. Cow III calved normally, and direct cultural examinations and guinea pig inoculation of colostrum and membranes were negative for Brucella. Cow IV failed to develop a significant agglutination response and was sold for slaughter. Unfortunately, tissues for bacteriologic examination were unobtainable. Cow V failed to show a significant agglutination response and calved normally with negative bacteriologic findings.

The results of blood examinations of the 2 gilts confined in close contact with cow V indicate active infection, and *Br. suis* was recovered from the blood stream on numerous occasions. One gilt developed an abscess near the mandible and *Br. suis* was recovered from this abscess. Both gilts farrowed normally. Four of 6 pigs farrowed by, and kept in the same lot with, these sows developed serum agglutination responses indicative of infection when tested as weanling pigs; presumably, therefore, active infection resulted from contact with the infected parent stock.

The results of examinations of milk from the third group of animals are shown in table 5. Of a total of 41 examinations of samples obtained from cow 0, *Br. suis* was recovered 34 times from the right front quarter, 37 times from the right rear quarter, and 15 times from the left front quarter (not exposed experimentally). The organism was isolated from the right quarters immediately after exposure, but was not recovered from the left front quarter until thirty-nine days later. Isolations continued up to the time of slaughter. Of 48 examinations of cow 1, 32 isolations were effected from the right front quarter and 42 from the right rear quarter. Isolations from cow 1 likewise continued up to the

time of slaughter. Of 36 examinations of the milk of cow 2, isolations of *Br. suis* began from the right front quarter on the second day after the second repeat exposure and totaled 19. From the right rear quarter, isolations began twenty-one days following the second repeat exposure. Isolations continued up to the time of withdrawal from the experiment, *i. e.*, approximately two months after *Br. suis* eliminations first began. Cow 3 began eliminating *Br. suis* on the second day after the second repeat exposure. Of 47 examinations, recovery was effected 13 times from the right front quarter and 14 times from the right rear quarter. Cow 3 ceased eliminating *Br. suis* approximately one month after infection.

Attempts to isolate *Br. suis* from the milk of cows 4 and 5 resulted in failure.

Agglutination responses and the results of cultural examinations of colostrum, blood, and parturient uterine discharges are shown in table 6. In all cases, agglutination responses indicative of active infection were elicited, with the possible exception of cows 4 and 5. Agglutination responses of cows 0, 1, 2, and 3 were not transient in nature, as in previous trials, but remained significantly high. It is interesting to note that cows 0 and 1 reacted positively at 1 : 2,560 and 1 : 5,120, respectively, within ten days after exposure. Later observations on the same animals revealed positive agglutination titers at 1 : 10,240 in each case.

In all cases of established active udder infection, gross pathologic changes in the infected quarters were observed. Grossly, the affected quarters exhibited considerable swelling and firmness, indicating an acute inflammation. At no time, however, were the animals observed to show systemic reactions. The observable external changes were accompanied by alterations in the udder secretions ranging from flaky milk to a serosanguineous secretion. Since the mastitis produced was not anticipated, no bacteriologic, microscopic, or chemical tests were conducted on the milk or udders of these animals prior to Brucella exposure, although physically the udders and the milk were apparently normal. However, postmortem cultural examinations of the affected quarters of cow 0 showed freedom from organisms other than *Br. suis*. Further, 2 additional animals (not included in the data reported here) when exposed to *Br. suis* by the intramammary route and destroyed seven and eleven days postexposure, respectively, showed freedom from organisms other than the one injected in the quarters showing acute mastitis on autopsy. As yet no histopathologic studies have been made to determine microscopic changes in the mammary tissues.

DISCUSSION

Although the experimental work reported would indicate that there is very little danger of the transmission of *Br. suis* from infected swine to cattle, nevertheless, it does occur occasionally in nature. The work demonstrates that the transient agglutination responses developed as a result of exposure of cattle to *Br. suis*-infected swine do not

TABLE 5—Results of Milk Examination of 6 Cows Exposed to Brucella Suis by Various Routes

Cow No.	Route of exposure	No. of exams	No. of isolations and organism recovered			
			RF	RR	LF	LR
0	Teat canal	41	34 *Br. suis*	37 *Br. suis*	15 *Br. suis*	None
1	Teat canal	48	32 *Br. suis*	42 *Br. suis*	None	None
2	Teat orifice	36	19 *Br. suis*	8 *Br. suis*	None	None
3	Teat orifice	47	13 *Br. suis*	14 *Br. suis*	None	None
4	Cutaneous	33	None	None	None	None
5	Cutaneous	33	None	None	None	None

RF = right front quarter; RR = right rear quarter.
LF = left front quarter; LR = left rear quarter.

TABLE 6—Blood Examination and Parturitional Results on 6 Cows Exposed to Brucella Suis by Various Routes

TABLE 6—Blood Examination and Parturitional Results on 6 Cows Exposed to Brucella Suis by Various Routes

Cow No.	Pre-exp. titer; blood culture	Route of exposure	Date of exposure and re-exposure	Agglutination titer; blood culture days after exposure								Parturition	
				10	14	28	35	45	71	100	128	141	Colostral culture	Cultural results‡
0	-----	Teat canal	10/27/47	+++++	+++++	+++++	+++++	+++++	+++++	+++++*			N.	Pos.	Not done
1	-----	Teat canal	10/27/47	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++*		N.	Pos.	Neg.
2	-----	Teat orifice	10/27/47 11/ 7/47 11/18/47	-----	-----	T----	+PT--	+++PT	++++P	++PT-	++PT-	+PT--	N.†	Inc.	Inc.
3	-----	Teat orifice	10/27/47 11/ 7/47 11/18/47	-----	-----	T----	+++++	+++++	+++++	+++++	+++++	+++++	N.	Neg.	Neg.
4	-----	Cut.	10/27/47 11/ 7/47 11/18/47	++PT-	+PT--	PT---	+PTT-	+PT--	PT---	T----	T----	T----	N.	Neg.	Not done
5	-----	Cut.	10/27/47 11/ 7/47 11/18/47	++PT-	++PT-	++PT-	++PT-	+PT--	++PT-	P----	-----	-----	N.	Neg.	Neg.

+ = complete agglutination; P = partial agglutination; T = trace of agglutination; Pos. = positive; Neg. = negative; N. = normal. Indicates calving was full term; Inc. = incomplete, indicates that studies have not been completed. *Removed for slaughter †Retained membranes. ‡At parturition. Cut. indicates application of *Br. suis* to abraded skin over left front and left rear quarters.

materially affect the degree of resistance to subsequent infection by *Br. abortus*, although the agglutination response and observed abortion rate were apparently decreased.

Under the conditions of the experiment, it has been definitely demonstrated that *Br. suis* infection can be established in the udder of the dairy cow. In such instances, the infecting organism was eliminated for comparatively long periods of time in the secretions of infected quarters. Indeed, such elimination apparently persists not only indefinitely but can assume an intermittent character. Such eliminations of *Br. suis* do constitute an important public health problem and must be considered a menace to consumers of such milk if ingested in the raw state, in spite of the comparative infrequency of such occurrences.

In view of these results, if the commonly accepted mode of transmission of bovine mastitis is correct, it seems plausible to hypothesize that *Br. suis* may gain access to the mammary gland by an ascending type of infection through the teat canal and establish itself in the actively secreting tissues.

It is interesting to note that here is additional experimental evidence that Brucella organisms may cause acute bovine mastitis. While these experiments can be justifiably criticised for the lack of pre-exposure examinations of the animals for mastitis, the authors suggest that the results observed be considered as strong circumstantial evidence in support of the likelihood that the acute mastitis observed in these experiments may have been the direct result of the experimental exposure to *Br. suis via* the mammary tissue. The previously mentioned delinquency in the planning and execution of these experiments has prompted repetition with experiments correcting this error. Such experiments are now in progress, and the results will be reported later.

SUMMARY

1) One of 9 cows exposed to *Brucella suis* either by natural contact with known infected swine or fed infected swine fetuses and membranes yielded a culture of this

organism at parturition (*see* table 1). Transient agglutination responses were demonstrated in the remaining 8 cows.

2) Exposure of 6 cows, previously exposed to *Br. suis*, to *Brucella abortus* failed to demonstrate resistence to infection, although the agglutination response and abortion rate were lower than in a group of controls.

3) Exposure of 5 cows by various routes resulted in active infection with the elimination of *Br. suis* in the milk in only 1 case.

This animal was exposed by introducing a live culture directly into the teat canal. The serum agglutination response rose to a titer of 1 : 400 within ten days and was not transient in nature.

4) Exposure of 2 additional cows by injection of a suspension of live *Br. suis* organisms directly into the teat canals also resulted in active infection with elimination of the organism in the milk. Exposure of 2 more cows, by applying viable *Br. suis* organisms to the open teat orifice, resulted in active infection of the quarters exposed.

5) Invasion of the mammary tissues under the conditions employed apparently resulted in acute mastitis of the quarters involved. While justifiable criticism may be advanced for the lack of pre-exposure examinations of the mammary glands for mastitis, the authors believe that the results observed should be considered as strong circumstantial evidence that the acute mastitis observed may have been the direct result of experimental exposure to *Br. suis via* the mammary tissue.

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Further Observations on Experimental Treatments of Trichomonas Foetus Infection in Bulls

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IN A PREVIOUS paper,[1] the literature pertinent to treatment of bulls infected with *Trichomonas foetus* was reviewed, and it was reported that ten infections in 8 bulls had been treated experimentally with 21 courses of potassium iodide (KI) orally and/or sodium iodide (NaI) intravenously and that six of these ten infections were terminated; also that 3 infected bulls had received 12 experimental treatments with six active trichomonadicidal agents applied as preputial douches or ointments and that no infections were terminated.

It is the purpose of the present paper to report additional observations on experimental treatment of trichomonad-infected bulls. In Part I, further experimental trials employing NaI intravenously and certain additional observations are reported. In Part II, experiments are reported in which three topically applied trichomonadicidal agents were employed, namely, silver-protein powder, trypaflavine in a water-soluble ointment base, and a German-developed and manufactured proprietary compound.

Part I. Intravenous Administration of Sodium Iodide

EXPERIMENTAL PROCEDURE

Eight bulls (274, 281, 283, 289, 290, 300, 301, and 302) infected with *T. foetus* were used in the present investigations. Seven had not been previously treated, and the eighth (274) had been unsuccessfully treated on three occasions, as previously reported.[1]

Treatment and observation of the first 5 bulls listed were carried out at the Zoölogical Division, Agricultural Research Center, Beltsville, Md.; the last 3 bulls were treated and observed at nearby Maryland dairy farm H. The technique employed in collection and examination of genital samples[2, 3] and the procedure followed in administration of NaI[1] have been previously described.

Further trials have been conducted with intravenously administered NaI, because, as previously pointed out, oral administration of KI requires daily handling of bulls for three to four weeks; the iodine* does not appear in the prepuce for more than a week and then only in limited, variable concentration; and the necessary period of iodism is longer and probably more severe. Based on the previously conducted experiments, the regimen selected for trial in the present experiments was: Five doses given intravenously at intervals of forty-eight hours, each dose consisting of 5 Gm. NaI/100 lb. of body weight, dissolved in 500 cc. of sterile distilled water.

RESULTS

Table 1 shows two successful and six unsuccessful treatments involving 8 infected bulls. Histories and post-treatment observations of the 2 cured bulls are related in the following protocols:

PROTOCOLS

Bull 281.—A purebred Aberdeen Angus bull, born July 3, 1943, was infected on arrival or became infected in Pennsylvania herd E. Its breeding history in that herd indicated it had been infected not less than six months.

Between July 10, 1946, and Aug. 15, 1946, trichomonads were found in all of six preputial samples examined.

Treatment.—On Aug. 20, 22, 24, 26, and 28, 1946, 75 Gm. of sodium iodide were administered intravenously.

Post-Treatment Obervations.—(1) Preputial samples were examined at least once each week from Aug. 21, 1946, until Jan. 8, 1947. Trichomonads were not found on any examination subsequent to the beginning of treatment (Aug. 20, 1946). (2) On Nov. 4, 1946, the bull served female 254, a virgin heifer. This coitus initiated a normal pregnancy that was terminated on Aug. 17,

This work was carried out at the United States Department of Agriculture, Agricultural Research Center, Beltsville, Md.

The author, at the time of this study, was from the Zoölogical Division, Bureau of Animal Industry, Agricultural Research Administration, United States Department of Agriculture, Beltsville, Md. He is now at the School of Veterinary Medicine, U. of Minnesota, St. Paul.

*The iodine compound is at present unidentified. Hereafter, in this paper, the word iodine is used to designate this unknown iodine compound.

1947, by the birth of a normal female calf. On Dec. 12, 1946, the bull served female 233, a virgin heifer. This coitus initiated a normal pregnancy that was terminated on Sept. 24, 1947, by the birth of a normal male calf. (3) Vaginal specimens from each of the 2 females were examined each week day from the respective days of first coitus through the postpartum periods. In no instances were trichomonads present. (4) This bull was returned to the herd from which it had originated on Jan. 8, 1947. Its breeding performance, which included service of many susceptible females, has been excellent.

Result.—Treatment of bull 281 was successful.

Bull 283.—A purebred Aberdeen Angus bull, born April 15, 1941, became infected while in Maryland herd F. The bull's breeding history in that herd indicates that it was infected several years before these experiments were begun. Diagnosis of infection was made first by Dr. Leo J. Poelma, Livestock Sanitary Service, University of Maryland, College Park, Md., during July, 1946. Between July and Aug. 15, 1946, trichomonads were found in both of two preputial samples examined.

Treatment.—On Aug. 20, 22, 24, 26, and 28, 1946, 70 Gm. of sodium iodide were administered intravenously.

Post-Treatment Observations.—(1) Preputial samples were examined at least once each week from Aug. 21, 1946, to Feb. 19, 1947. Trichomonads were not found at any examination subsequent to the beginning of treatment (Aug. 20, 1946). (2) On Oct. 30, 1946, the bull served female 253, a virgin heifer. This coitus initiated a normal pregnancy that was terminated on Aug. 3, 1947, by the birth of a normal male calf. On Dec. 31, 1946, the bull served female 255, a virgin heifer. This coitus initiated a pregnancy that was diagnosed by palpation *per rectum* on Feb. 1, 1946, and confirmed at a later date. On April 15, 1946, palpation revealed a nonpregnant uterus. (This heifer was subsequently proved susceptible by inoculation on May 8, 1947, with preputial flushings from an infected bull; the animal developed a typical infection.) (3) Vaginal specimens from female 253 were examined on each week day from the day of coitus through the postpartum period. In no instance were trichomonads present. Vaginal specimens from female 255 were examined and found negative on each week day from

TABLE I—Experimental Treatments of Trichomonas Foetus Infection in Bulls with Intravenously Administered Sodium Iodide

Bull No.	Trial No.	Weight	Dose and date of treatment	Result	Time of appearance of *T. foetus* after treatment
274[a]	4	1750	85 Gm., Aug. 20, 22, 24, 26, and 28, 1946	F.	4th wk.
281[b]	1	1520	75 Gm., Aug. 20, 22, 24, 26, and 28, 1946	S.	
283[c]	1	1455	70 Gm., Aug. 20, 22, 24, 26, and 28, 1946	S.	
289[d]	1	1640	80 Gm., Feb. 24, 26, 28, Mar. 1, and 3, 1946	F.	7th wk.
290[e]	1	1810	90 Gm., Feb. 24, 26, 28, Mar. 1, and 3, 1946	F.	5th wk.
300[f]	1	Est. 1400	70 Gm., Nov. 14, 16, 18, 20, and 22, 1946	F.	11th wk.
301[g]	1	Est. 1500	75 Gm., Nov. 14, 16, 18, 20, and 22, 1946	F.	7th wk.
302[h]	1	Est. 1600	80 Gm., Nov. 14, 16, 18, 20, and 22, 1946	F.	9th wk.

[a]Purebred Aberdeen Angus from Maryland herd D, born March 22, 1937. Previous treatments in protocol.[1]
[b]Purebred Aberdeen Angus from Pennsylvania herd E, born July 3, 1943.
[c]Purebred Aberdeen Angus from Maryland herd F, born April 15, 1941.
[d]Purebred Aberdeen Angus from North Dakota herd G, born June 13, 1941.
[e]Purebred Aberdeen Angus from Maryland herd F, born Nov. 6, 1942.
[f]Purebred Guernsey from Maryland herd H, bornDec. 31, 1939.
[g]Purebred Guernsey from Maryland herd H, born Jan. 25, 1937.
[h]Purebred Guernsey from Maryland herd H, born late 1941.
F. = Failure; S. = Success.

the day of coitus up to the time of intentional exposure. (4) This bull was returned to the herd from which it had originated on Feb. 19, 1947. Its subsequent breeding performance, which included service of many susceptible females, has been excellent.

Result.—Treatment of bull 283 was successful.

DISCUSSION

Sippel, Bassham, and Bennett[4] have reported cure of 2 trichomonad-infected bulls by intravenously administered NaI. Considering their cases, those previously reported by the writer,[1] and those reported herein, a total of 19 infections in 17 bulls have now been treated by administration of iodides. Ten infections have been terminated, two by orally administered KI and eight by intravenously administered NaI. A total of 31 courses of treatment have been administered.

Thirteen infections received only one course of treatment. Eight of these infections were cured; the remaining five infections were not re-treated with iodides. Six infections received more than one treatment, two, three, and four treatments being administered to 2 bulls each. One infection was terminated by the third course of treatment and one by the fourth.

In considering possible causes of failure to cure some bulls, there is no clear cut evidence for or against iodide fastness playing a part. However, the fact that 2 bulls were cured by re-treatments, 1 by the third treatment and 1 by the fourth treatment, suggests that drug fastness is not a significant consideration.

An interesting and rather striking seasonal correlation of successes and failures has been noted. Its true significance and validity are yet to be ascertained. Table 2 shows all iodide treatments administered,

TABLE 2—Distribution by Months of All Iodide[5] Treatments

Bull No.	Jan.	Feb.	Mar.	April	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
193							S (1)					
207	F (1)									F (2)		
208						S (1)†						
208*					F (1)					F (2)		
214					S (1)†							
214*	F (1)					F (2)				F (3)		
250		S (1)										
266			F (2)‡				S (3)				F (1)	
274								F (4)		F (3)	F (2)	F (1)
275			F (2)‡			S (4)				F (3)		F (1)
281								S (1)				
283								S (2)				
289		F (1)										
290		F (1)										
300											F (1)	
301											F (1)	
302											F (1)	
A**							S (1)					
B**							S (1)					
Totals of success and failures, months	2F	2F;1S	2F		1F;1S	1F;2S	4S	1F;2S		5F	5F	2F

F = Failure; S = Success; (1), (2), (3), (4) = number of treatment.
*Second infection.
**Cases reported by Sippel, Bassham, and Bennett.[4]
†KI orally.
‡NaI intravenously followed by KI orally.
All treatments NaI intravenously except those designated † and ‡.

including those of Sippel, Bassham, and Bennett,[4] arranged in accordance with the months in which treatments were begun. The greater proportion of successful outcomes occurring during the warm to hot months of March through August (9 successes in 12 trials) contrasts markedly with the results occurring during the cool to cold months of October through March (1 success in 19 trials). It has been shown that iodine appears in the prepuce in chemically detectable concentration following oral and intravenous administration of iodides. In order to better understand the mechanism of this experimental treatment, its failures, and the possible relation to its efficacy of environmental temperatures, determination of the route of iodine elimination is essential. Further studies on the physiology and histology of the preputial membranes are indicated. It must be ascertained precisely whether the iodine found in the preputial cavity reaches that location (1) *via* the urine, (2) through the genital membranes, or (3) by both routes.

Histologic sections were made from several areas of the prepuce and glans penis of 3 bulls with the object of determining the anatomic classifications of the preputial membrane. After histologic examination of these sections and on reviewing certain literature, Dr. Herman R. Seibold of the Pathological Division, Bureau of Animal Industry, reported as follows:

> All of the sections of prepuce and glans showed a superficial layer of non-cornified, stratified, squamous epithelium. No mucous secreting cells (goblet cells) were present, and there were no glandular structures in the underlying connective tissue of the sections examined.
>
> *Remarks:* According to Sisson, the lining membrane of the prepuce of the bull is covered with squamous, stratified epithelium and has coiled tubular glands. (These glands were not found in the limited amount of material examined by us.) The penile layer of the prepuce is glandless.
>
> Trautmann and Fiebiger state that the parietal layer of the prepuce consists of a papillary body and stratified, squamous epithelium. In most animals, the hair, as well as the sebaceous and sweat glands, is lost shortly behind the preputial orifice ('bald vom Ostium Praeputiale ab') where the glands are well developed, and sometimes even free sebaceous glands opening on the surface are present. The remainder of the parietal layer of the prepuce is free of hairs and glands. The membrane, which covers the glans, is also hairless and gland-free.
>
> Regarding the anatomical classification of the preputial lining:
>
> 1. Sisson states that, in the horse, the skin of the prepuce within the preputial ring resembles a *non-glandular mucous membrane.*
>
> 2. Strangeway states that the inflected fold of the prepuce (in animals) is void of hair and resembles that of the glans in structure, *being intermediate between that of skin and mucous membrane,* etc.
>
> 3. Chauveau states, in general, that lining integument of the prepuce—holds a middle place, with regard to organization, between the skin and mucous membranes. In the bull, the free portion of the organ (penis) is covered by a fine, papillated, very sensitive,*rose-colored mucous membrane.*
>
> 4. According to Maximow, the inner surface of the prepuce in man is moist and has the *character of a mucous membrane.*
>
> 5. Piersol states that the skin lining the prepuce (in man) and covering the glans is modified so that *it somewhat resembles a mucous membrane, as it is often inaccurately described.*
>
> 6. Böhm-Davidoff and Huber state that, in man, the prepuce is a duplication of the skin, *the inner surface presenting the appearance of a mucous membrane.*
>
> As noted above, we were unable to identify glandular structures in the sections of glans and prepuce submitted. Examination of the literature shows that glands in the prepuce of different species are generally described as 'sebaceous,' 'modified sebaceous,' 'coiled tubular,' and 'sweat' glands. This information lends support to the concept that the lining of the prepuce and the covering of the glans penis is a modified integument rather than a mucous membrane.

The chemical form and the manner in which iodine reaches the preputial cavity are at present unknown. However, relative to the latter, there is suggestive evidence that the genital membrane may be a secretory structure. On postmortem examination of a bull in which a permanent, perineal, urethral fistula had been established surgically more than a month previously (there being no possibility of flow of urine through the distal urethra), the genital membrane was found to be moist and otherwise normal in appearance.

Despite the fact that trials with iodides have not as yet resulted in as favorable a

ratio of cures as the method described in Part II of this paper, further investigations are justified. The intravenous NaI method has several distinct advantages: (1) A technique already mastered by practitioners is employed; (2) no special equipment and a minimum of handling of the subject are necessary; and (3) it is a faster and generally safer procedure for the operator and his helpers, as well as for the bull.

Part II. Direct Application of Trichomonadicidal Agents to the Genital Membranes, Especially a German-Developed Proprietary Compound*

In continuation of our investigation of treatments for trichomonad-infected bulls with topically applied trichomonadicidal agents, silver-protein** in the form of a powder by intrapreputial insufflation, trypaflavine in a water-soluble base† by direct application to the genital membranes, and G.P.C. by direct application to the genital membranes, have been tried experimentally. Use for this purpose of the first two was new. Use of G.P.C., until recently known only as a trypaflavine-containing ointment, was reported separately by Abelein[5,6] and Swangard[7,8,9] for treatment of trichomoniasis of bulls. While the animals were cast and under epidural anesthesia, this oinment was applied to the preputial membranes and the anterior urethra douched with 0.1 per cent trypaflavine solution. Abelein's experimental results have already been reviewed.[1] Recently, Vandeplassche[10] reported cure of 3 of 4 infected bulls with this same product.

Strangely, the composition of G.P.C. was unknown to Abelein and to Swangard at the time of their respective reports. During the recent war, it was impossible for us to obtain either the product or information relative to its composition. As previously reported,[1] an ointment thought similar to that used in Germany was furnished by Dr. Swangard, then of Eugene, Ore. This ointment was subjected to a single experimental trial with unsuccessful outcome.

On April 11, 1946, on request of the chief of the Bureau of Animal Industry, Dr. Swangard, proceeding to Germany under auspices of the Technical Industrial Intelligence Division of the Department of Commerce, was directed by that Division to investigate G.P.C. and arrange shipment of sufficient quantity of the material to Beltsville for experimental use. Its composition and manufacture was the subject of a comprehensive report subsequently prepared by Dr. Swangard.[11]

EXPERIMENTAL PROCEDURE

Eight bulls (207, 208, 274, 289, 290, 300, 301, and 302), injected with *T. foetus*, were used in the present investigations. Each had been previously treated on at least one occasion with iodides or topically applied trichomonadicidal agents.

Treatment and observation of bulls other than 300, 301, and 302 were carried out at the Zoölogical Division, Agricultural Research Center, Beltsville, Md.; the latter 3 bulls were treated and observed on nearby Maryland dairy farm H. The techniques employed in collection and examination of genital samples have been previously described.[2, 3]

In the trial in which a silver-protein powder was used, a satisfactory insufflator was made by modifying an ordinary human vaginal insufflator. An extension of about 18 in. of ¼-in. diameter stainless steel tubing was welded to the original nozzle. Instead of the

*Hereafter designated in this paper as G.P.C. This product was sold under the trade name of bovoflavin-salbe by Behringwerke, I. G., Farbenindustrie Aktiengesellschaft, Leverkusen, Germany, and later by Farbwerke Hoechst, Frankfurt (M)—Höchst, Germany.

According to the label, "bovoflavin-salbe" is composed of 0.5 per cent 3, 6-Diamino-10-methyacridiniumchlorid (neutral trypaflavine‡) and 0.05 per cent Bis-2-methyl-4-amino-chinolyl-6-carbamid-hydrochlorid (surfen‡) in a fat-free, washable, ointment base.

According to Swangard,[11] bovoflavin-salbe is composed of:

Trypaflavine (mixture of 3-6-diamino-10-methylacridinium-chloride and 3-6-diamino-acridinechlorhydrate)	0.5 kg.
Surfen A (di-2-methyl-4-amino-quinolyl-6-malic-acid-amide)	0.05 kg.
Tryethyleneglycol	55.00 kg.
P.60 (condensation product of ethylene oxide with water, $HO\ CH_2O(CH_2CH_2O)_{38}$-$CH_2CH_2OH$)	38.45 kg.
Aqua. dest.	6.00 kg.

The Office of the Alien Property Custodian should be consulted to ascertain the legal status of the individual ingredients and combination thereof, known as bovoflavin-salbe, before this product is manufactured or offered for sale in the United States.

The interest, effort, and coöperation of the Department of Commerce, particularly Mr. John C. Green, and Drs. W. M. Swangard, C. E. Reid, and A. Viehoever, in obtaining from Germany quantities of bovoflavin-salbe and pertinent information are gratefully acknowledged.

**Argyrol (Barnes).
†Carboxymethocel-S (Dod).
‡Name inserted by author.

original hand bulb, an ordinary tire pump was connected to the instrument, using a long piece of plastic tubing. Mechanically, this apparatus functioned ideally. Bull 274, confined in stocks, received 18 applications on successive Mondays, Wednesdays, and Fridays (May 22, 1946, to July 1, 1946) with 1½ oz. of powder composed of silver protein§ 40 per cent, kaolin 30 per cent, and *beta* lactose 30 per cent.

In a trial using 0.5 per cent trypaflavine in a water-soluble gelatinous base,‖ the first application was carried out by Dr. Swangard and the second by the writer. Bull 208 was given epidurally 40 cc. of 1 per cent procaine, cast, and secured with ropes. On April 21, 1946, 100 cc. of the ointment was rubbed thoroughly into the membranes and 10 cc. of 0.1 per cent solution of trypaflavine injected into the urethra. Application was repeated approximately ten days later.

All applications of G.P.C. were made while bulls were in the standing position confined in stocks; no other physical restraint was necessary. It had previously been found that properly designed stocks were imperative for the success of the procedure employed. The stocks were strongly constructed and had stanchion bars that could be tightly clamped on the neck,* preventing all forward or backward movement of the bull. The width of the stocks at the level of the pelvis was such that all lateral movement was prevented. Twenty-eight inches was an ample width for all except the largest bulls. When treating small bulls, 2 by 4's were inserted medially to the vertical members of the stocks to take up space.

Clean sand was spread under the bull's hind feet to prevent slipping, the effects of the anesthetic preventing full control of the hind legs. Invariably, bulls stood with their feet farther forward than natural.

After the area of puncture was shaved, scrubbed, and disinfected, 15 to 20 cc. of 1 per cent procaine with 0.1 per cent epinephrine was injected very slowly (up to 5 minutes) between either the sacrum and first coccygeal vertebra or between the first and

§Argyrol (Barnes).
‖Carboxymethocel-S (Dow).

TABLE 3—Experimental Treatments of Trichomonas Foetus in Bulls with G.P.C.

Bull No.	Trial No.	Dates of applications	Post-treatment results: Examination of preputial samples	Post-treatment results: Coitus with susceptible test females: Reproduction	Post-treatment results: Coitus with susceptible test females: Examination of vaginal samples	Herd test	Result of treatment
207	1	8/ 2/46 8/12/46	Neg. weekly to 2/6/47.	†249, 10/25/46; normal calf, 8/4/47.	Neg. each weekday	N.	S.
208	1	8/ 2/46 8/12/46	Neg. biweekly to 2nd week (8/21/46); pos. thereafter.	...	...	...	F.
208	2	5/12/47 5/22/47	Neg. biweekly to 14th week (8/25/47); pos. thereafter.	...	...	...	F.
274	1	10/17/46 10/28/46	Neg. weekly to 4/25/47.	†262, 1/16/47; normal calf, 10/25/47. †252, 3/9/47; preg. on slaughter.	Neg. each weekday Neg. each weekday	E.	S.
289	1	5/12/47 5/22/47	Neg. biweekly to 8/7/47.	†241, 7/3/47; preg. as of 3/24/48. †659, 8/26/47; status undetermined.	Neg. each weekday Neg., 9/12/47	F-inc.	S.
290	1	5/13/47 5/22/47	Neg. biweekly to 10/6/47.	†278, 7/27/47; preg. as of 3/24/48. †280, 8/26/47; preg. as of 3/24/48.	Neg. each weekday Neg. each weekday	F-inc.	S.
300	1	3/26/47 4/ 7/47	Neg., 5/26/47, 6/4/47, 6/25/47, 7/1/47, 8/7/47.	†86158, 7/15/47; preg. as of 2/3/48. †91535, 8/16/47; preg. as of 11/13/47.*	Neg., 7/25/47, 8/1/47, 8/7/47 Neg., 8/28/47, 9/4/47	F-inc.	S.
301	1	3/26/47 4/ 7/47	Neg., 5/26/47, 6/4/47, 6/18/47, 7/1/47, 8/7/47.	†86156, 7/7/47; preg. as of 2/3/48. †86161, 8/15/47; preg. as of 2/3/48.	Neg., 7/25/47, 8/1/47, 8/7/47 Neg., 8/29/47 9/4/47	F-inc.	S.
302	1	3/26/47 4/ 7/47	Neg., 5/26/47, 6/4/47, 6/18/47, 6/25/47, 7/1/47, 8/7/47.	†86160, 7/11/47; not preg. †86165, 8/12/47; preg. as of 2/3/47.	Neg., 7/25/47, 8/1/47, 8/7/47 Neg., 8/29/47 9/4/47	F-inc.	S.

*Found not pregnant, 1/6/48; extremely debilitated; sold.
N = none; F-inc. = favorable-incomplete; S = success; F = failure; E = excellent.
†Female.

second coccygeal vertebrae. After twenty to thirty minutes, it was possible to cause protrusion of the glans to a position where it could be grasped and held; usually, some manipulation was necessary.

After the genital membranes were thoroughly washed with soapy water, rinsed, and dried, 120 cc. of G.P.C was thoroughly rubbed for fifteen minutes into the surface of the glans and prepuce. The urethra was injected with 15 to 20 cc. of 0.1 per cent trypaflavine solution and then the urethral orifice held for five minutes.

Each experimental treatment consisted of two applications ten to fourteen days apart.

RESULTS

Insufflation of the preputial cavity twice weekly for six weeks with silver-protein powder failed to effect a cure in 1 trichomonad-infected bull. *Trichomonas foetus* were found at three of seven examinations during the period of treatment as well as subsequent to treatment.

Two topical applications to the genital membranes of trypaflavine in a water-soluble gelatinous base failed to effect cure of 1 trichomonad-infected bull. *Trichomonas foetus* were found during the first week post-treatment and subsequently.

Post-treatment tests and results of application of G.P.C. are shown in table 3. Eight bulls were treated; 7 were cured by a single treatment (2 applications), and 1 was not cured despite two treatments (4 applications).

DISCUSSION

A) *Principles in Treatment.*—While observing trichomonad-infected bulls and while treating under experimental conditions, certain facts warranting consideration before undertaking treatments and governing the carrying out of treatments under practical conditions have become apparent:

1) Bulls are either infected or not infected with *T. foetus*; there are no "slight cases" or "touches" of trichomoniasis. The infection is never acute and is no different when initiated than after it is well established. However, the level of trichomonad numbers in the prepuce of infected bulls, as measured by regular examination of preputial samples, fluctuates considerably. Several *T. foetus* may be present in each low power microscopic field, or a few or only one may be found in an entire sample. Frequently, no *T. foetus* can be found in several successive samples from an infected bull.

2) Treatment of trichomonad-infected bulls under practical conditions is pointless, unless eradication of *T. foetus* from female members of the herd is concomitantly accomplished by installing a system of hygienic breeding.[12] Recovered bulls are susceptible to reinfection.

3) Although treatment of trichomonad-infected bulls is still in the experimental stage, field trials have been a part of our experimental series. From practical experience, it is obvious that treatment of trichomonad-infected bulls by any method is a long, exacting, and costly procedure, requiring frequent handling and observation of the subject over a period of six months or more by a veterinarian thoroughly familiar with trichomoniasis. Under practical conditions, infected bulls not of exceptional value as sires should be slaughtered promptly.

4) At least until the efficacy of treatments for trichomonad-infected bulls has been clearly established by extensive trials involving large numbers of infected bulls, meticulous observation of bulls post-treatment is imperative.

5) For a period of approximately three months post-treatment, negative preputial samples have little significance, even if collected and examined biweekly. Test matings with susceptible females, likewise, are not reliable during this period. Unsuccessful treatments are typically not revealed by reappearance of *T. foetus* in samples for one to two months post-treatment. One bull (208), negative on biweekly examinations for thirteen weeks post-treatment, failed to infect a virgin heifer with which it had coitus during this period but was found positive after the fourteenth week.

6) Post-treatment evaluation of the effectiveness of treatment is dependent upon many examinations over a considerable period of time. It must comprise:

1) Examination of Preputial Samples.—

Samples should be collected and examined frequently, weekly if possible, during the fourth, fifth, and sixth months post-treatment. Further examinations are desirable. Negative samples occurring during the first three months post-treatment are of little significance.

2) Test Matings with Virgin Heifers.—Coitus with two or more virgin heifers should be permitted at intervals during the fourth, fifth, or sixth months post-treatment, and vaginal samples should be examined between the twelfth and nineteenth day post coitus. Examinations for pregnancy should be made at appropriate times.

3) Extended Observations.—In the field, careful check should be kept of the bull's efficiency when restored to service. When afforded, opportunity should always be taken to make further examinations of the bull, as well as those females with which it has coitus.

B) G.P.C.—Although the present series of trichomonad-infected bulls treated experimentally with G.P.C. is comprised of too few trials to determine critically the efficacy of this material, outstanding merit is clearly evident. This is the only material topically applied which has, in our experiments, cured infected bulls.

It seems probable that its effectiveness is derived from its constituents other than trypaflavine, trypaflavine itself being the least specific, important constituent. The other ingredients presumably permit extraordinary contact with, and penetration of, membranes. Several other trichomonadicidal agents decidedly superior to trypaflavine on *in vitro* tests have failed to effect cures when employed with different ointment bases. It seems logical that these ointment bases were unsatisfactory and were primarily responsible for the failures rather than the active, trichomonadicidal agents.

Nevertheless, in retrospect, a possible flaw in our reasoning becomes apparent. Although the various agents employed unsuccessfully as douches appear to have been adequately tested (on 3 infected bulls, 2 of which were subsequently cured by other methods), the several experimental ointments employed unsuccessfully may not have been subjected to adequate trial. Five such trials were unsuccessful, but four of these involved infected bull 208, the same bull that subsequently resisted two courses of treatment with G.P.C. Three of these unsuccessful treatments were for its first infection, ultimately terminated with iodides; and one was for the bull's second infection, which resisted treatment with iodides as well as with G.P.C.

Generalization based on our limited experiments alone would be folly, but, in consideration of them and the reported work of others, it seems safe to conclude that: (1) This product is unique in its therapeutic properties in trichomonad-infected bulls; (2) its effectiveness is dependent largely upon its constituents other than trypaflavine; and (3) the many topically applied medicaments we and certain others have tried experimentally have no, or relatively little, value in treatment of trichomonad-infected bulls.

SUMMARY

1) Two of eight previously unreported experimental treatments of trichomonad-infected bulls with intravenously administered sodium iodide were successful.

2) Reports now exist of a total of 19 infections (17 trichomonad-infected bulls) having been treated experimentally with a total of 31 courses of iodides. Ten infections were terminated.

3) Intrapreputial insufflation with a silver-protein powder and direct application to the genital membrane of trypaflavine in a water-soluble ointment base each failed to cure 1 trichomonad-infected bull.

4) Seven of 8 trichomonad-infected bulls treated experimentally were cured by direct application to the genital membrane of a German-developed, proprietary compound.

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A Method of Collecting Sterile Blood from Cattle

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ON OCCASION, it is desirable to obtain a quantity of sterile blood from the bovine species. Especially in the field of research, where it is often imperative that sterile blood be drawn, workers have experienced considerable difficulty in obtaining a sample free of contaminating organisms. In fact, the relative rarity of success in obtaining sterile, bovine blood has prompted some wrongly to suggest certain microörganisms as being normal inhabitants of the blood stream of cattle. The difficulty is apparently related to the ineffectiveness of common cleansing and antiseptic techniques in freeing the bovine skin of its contaminating organisms at the site of operation. The author has developed a technique which has proved workable and requires very little in the way of preparation and equipment.

An area about 3 in. by 4 in. over the jugular groove is shaved or clipped. The area is then washed thoroughly with a soapy disinfectant. After drying with a towel, tincture of iodine is applied to the operative field and allowed to dry. A 14-gauge, 1-in. hypodermic needle is then introduced through the skin but not into the jugular vein. An 18-gauge, 3-in. needle is introduced through the lumen of the larger needle and this inner needle is then used to puncture the jugular vein after which the desired quantity of blood is removed. To facilitate ease of operation, a tourniquet is applied (ordinary rope) around the neck of the animal to distend the vein. The needles are sterile and the 18-gauge needle is attached to a sterile syringe into which the blood is drawn. After the desired quantity of blood is withdrawn, the needles are removed, the 18-gauge needle detached from the syringe, and a new sterile needle attached to the syringe for emptying the blood, into a suitable sterile container.

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The Brucella Abortus Ring Test

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IN 1937, the *Brucella abortus* (abortus-Bang*) ring test was introduced by Fleischhauer. Now, eleven years later, this ring test is a highly important factor in the collective combating of brucellosis in Denmark.

The ring test is performed by adding 1 drop of hematoxylin-stained Brucella antigen to 1 cc. of milk. Upon shaking thoroughly, the sample assumes a bluish violet color. It is incubated at 37 C., and the reading is made at the end of one hour, when the reaction, as a rule, will be complete. If the samples are incubated for a longer time, a decolorization will set in, induced by growth of bacteria, since the oxidized hematoxylin used for dyeing the antigen acts as an oxidation-reduction indicator.

The strength of the reaction is graduated as follows:

0 or ÷: Negative reaction. The cream layer, white; the column of milk below, bluish violet.

1 or (+): Doubtful reaction. The cream layer of approximately the same bluish violet color as the milk column below.

2 or +: Weakly positive reaction. Bluish violet colored cream layer forming a ring, the milk column not visibly decolorized.

3 or ++: Positive reaction. Bluish violet colored cream layer forming a ring, the milk column below perceptibly, but not totally, decolorized.

4 or +++: Strongly positive reaction. Bluish violet cream layer, distinctly separated, the milk column below entirely decolorized or white.

A circular dated Nov. 1, 1947, concerning the registration of brucellosis-free herds provides that the control of the registered herds may be carried out by means of ring test examinations alone. It also permits a herd to be registered as free of brucellosis on the basis of three consecutive negative ring tests of milk can samples, performed at intervals of about four months, and one negative blood test performed on all the cattle 1 year or more of age in the herd, from three to six months after the latest ring test.

THE SCHERN-GORLISCH REACTION

In order to fully appreciate this rapid development, it will be expedient to follow the evaluation of the ring test as a diagnostic means through the past years. Before doing so, I will outline briefly the Schern-Gorlisch reaction,[8] and how the ring test has been performed and graduated (standardized) in this country during the last three to four years. A critical survey of the available literature shows that the ring test antigen employed has, as a rule, been more or less nonspecific with the resultant effect that the so-called "positive" ring test reactions have in reality been a combination of a ring test reaction and a Schern-Gorlisch reaction.

The Schern-Gorlisch reaction is used to determine whether milk has been heat-treated. One cubic centimeter of milk is mixed with 1 drop of a 2 per cent suspension of red blood corpuscles, or a 1 per cent suspension of bone black, and incubated. The reading is made after incubation for two hours. If the milk sample is raw, the cream-rising capacity will persist, and a red or black ring, respectively, is formed on top as a consequence of the "indicator" (blood corpuscles or bone black) having been adsorbed to the fat globules and carried with these to the cream layer. However, the cream-rising capacity depends on the content of fat as well as on the size of the fat globules and on the nature of the protein membrane around the fat droplets. These factors vary from one animal to another. Hence, the Schern-Gorlisch reaction gives

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*The author, throughout the manuscript and references, used "abortus-Bang" but in accordance with the style of the AVMA journals, *Brucella abortus* has been substituted.

the most reliable result with pooled milk, in which individual variations will have been offset to some extent—exactly as in the ring test.

LITERATURE

Evolution of the Ring Test from 1937 *to* 1944, *Showing that the Antigen Used Has Been More or Less Nonspecific.*—The ring test was described for the first time in 1937 by G. Fleischhauer.[4] Subsequently, a series of German articles on the ring test was published in the years 1938 to 1940 by Fleischhauer and Herman,[5] Smitmanns,[11] Hermann,[6] and Seelemann and Mantovani.[9] These observers made comparative studies of the ring test and the slow agglutination test on whey. They found a far-reaching agreement between the two tests—the ring test gives more positive reactions on herd samples than the milk serum agglutination test, although the two tests are approximately equally sensitive when applied to milk from individual cows (as the lowest reaction value for positive agglutination the titer + + 1:5 has been used.)

Fleischhauer stated in 1937 that, in the examination of herd samples, only those reactions can be considered positive which appear after incubation for twenty minutes at the most, since most herd samples already produce a distinct formation of a ring after incubation for forty minutes, and the reaction therefore cannot be considered specific. One year later, Smitmanns claimed that reactions on herd samples can only be considered positive if they begin before the incubation has lasted for twenty-five minutes, and if after incubation for two hours they show a 2 to 4 mm. bluish violet ring on top, sharply separated from the dead-white milk below. Fleischhauer and Hermann, in the same year, ascribed reactions setting in after incubation for more than thirty-minutes to a Schern-Gorlisch reaction. In 1940, Seelemann and Mantovani[9] reported that, in definitely negative milk samples, a narrow, bluish violet ring generally appears after incubation for one hour, although without decolorization of the milk column below.

At the same time, they noted that the reliability of the ring test depends essentially upon the care with which the antigen is produced.

In 1943, Norell and Olson[7] published an article in Sweden on the value of serologic milk examinations by means of the *Brucella abortus* ring test. The ring test antigen which they used was evidently the best produced so far, as they were able to incubate the herd samples for fifty minutes, apparently without obtaining a particularly great number of nonspecific reactions. By comparative studies of the ring test and the slow agglutination test on whey (lowest reaction value, the titer 1:2.5) on 1,000 herd samples, agreement is found between 651 negative reactions and 215 positive reactions (total 866). Of the remaining 134 samples, 98 gave positive reactions to the ring test but negative reaction to the agglutination test. The remaining 36 suspicious herd samples gave doubtful ring test reactions; of these, 28 were recorded as negative, while eight showed the titer 1:2.5 to the whey agglutination test. Assuming the antigen employed to be specific, the doubtful ring test reactions will necessarily increase with a rising percentage of infection in the milk samples examined. If, therefore, the 36 doubtful ring test reactions are correlated with the 313 positive reactions, they represent 11.5 per cent of these. The ring test proved to be far more sensitive on herd samples than the whey agglutination test. In another study on 566 herd samples, Norell and Olson found, by means of the ring test, that 314 samples gave a negative reaction, 45 samples doubtful reactions, and 207 samples positive reactions. The doubtful reactions represent approximately double the percentage (21.7:11.5%) found in the first group studied, presumably because the ring test antigen employed in the latter study had been less specific.

In order to evaluate the applicability of the ring test as a diagnostic agent in combating brucellosis, they collected results of ring tests and blood tests on individual animals and on herd samples.

For the first mentioned category, a group of 453 cows was studied. As the lowest reaction value for the blood tests, the complete blood agglutination titer 1:20 was employed. If the doubtful reactions with the ring test are included in the negative reactions, of 453 cows, 338 gave negative reactions with the ring test, 26 of which gave positive reactions with the blood test, while 115 cows gave positive reactions with the ring test, 103 of which were positive with the blood test, and 1 doubtful. The ring test, correlated with the blood test, thus failed in 26 out of 129 cases (20.2%), while it gave positive reactions in 12 cases in which the blood test had been negative, corresponding to 9.3 per cent of the 129 cases in which the blood test had given positive reactions. It would thus seem that, for the purpose of detecting Brucella-infected animals, the blood test is more sensitive than the ring test despite the fact that the lowest reaction value for positive reaction has been put at the agglutination titer 1:20 total.

In the category of comparative examinations of herd samples and blood tests on the individual animals, Norell and Olson reported on 225 herds. In these herds, agreement was obtained in 213 cases (94.6%). In the remaining 12 cases, the ring test gave positive reactions in 8 cases, while the blood tests gave negative reaction; in the other four herds, the ring test was negative while the blood test was positive. Unfortunately, there is no information about the percentage of Brucella infection in this material, as with a specific ring test antigen in a completely Brucella-free environment a 100 per cent agreement would be expected.

Norell and Olson indicate that they have been able to dilute the milk from Brucella-

infected herds with Brucella-free milk up to 40 times without the reaction disappearing.

Prompted by these rather encouraging results, Danish investigators took up the study of the ring test. The result was a series of articles on the subject, by Winther and Hansen,[13] Seit and Leth Jorgensen,[10] the author, and Darnell.[3]

Winther and Hansen have undertaken the reading of the ring test after incubation for thirty minutes, claiming that reading after more than thirty minutes will give numerous reactions with typical formation of a ring and, in some cases, some decolorization, which must decidedly be interpreted as nonspecific. Winther and Hansen have principally undertaken comparative examinations of the ring test and the slow agglutination test on whey, partly on herd samples and partly on samples from individual cows. By examination of 548 herd samples, 414 samples are found to give positive reactions with the ring test, while 379 were positive to the whey agglutination test. The ring test has thus shown 6.4 per cent more positive reactions than the agglutination test. In 6 cases, they consider that the ring test has given an erroneous result. By examination of 254 samples from individual cows they find 31 to be positive with the ring test, while only 15 gave positive reactions with the whey agglutination test (lowest reaction complete at 1:2.5). The ring test has given twice as many positive results as the whey agglutination test, while previous examinations show that with incubation for thirty minutes the ring test usually gives about 10 per cent more positive reactions on pooled milk than does the whey agglutination test, and that the two methods are practically equally sensitive in the case of samples from individual cows. This seems to indicate that the ring test antigen employed in the last mentioned examinations, as far as specificity is concerned, has not been nearly equal to that used in the first mentioned examinations.

Winther and Hansen conclude that evidently nonspecific reactions only occur among weakly positive and doubtful tests, whereas very strong positive and absolutely negative reactions must be considered safe.

MATERIALS AND METHODS

Encouraged by Norell and Olson's studies, the author set to work with comparative studies between the *Brucella abortus* ring test and the slow agglutination test on milk and blood, in order to determine whether the ring test could be considered specific. The material used was six large, chronic, Brucella-infected herds in which mastitis and brucellosis programs, including calfhood vaccination and blood testing, had been running for a number of years. The samples of milk from individual cows and the blood samples corresponding to these were collected on the same day, and the herd samples (from milk cans, each representing 6 to 12 cows) were collected at the dairy the day following the collection of the previously mentioned samples; they thus correspond completely to each other.

The preliminary examinations showed that this ring test antigen apparently reacted satisfactorily, and it was placed at the disposal of the Danish State Veterinary Directorate, on behalf of which Seit and Leth Jorgensen set to work with the ring test examinations at Bornholm. All herds in that island, throughout the preceding seven years, had been blood-tested regularly and thus constituted excellent material for illustrating the value of the ring test as a diagnostic agent. Seit and Leth Jorgensen incubated their ring tests at 37 C. for forty minutes. The material comprised 3,362 herds, 2,768 of which showed concordant negative and 465 concordant positive results by comparative examinations of the ring test on herd samples and blood tests of individual animals. No less than 3,233 herds, or 96.2 per cent of the total material, thus showed agreement. Fifty-seven herds gave negative blood tests but positive ring tests, while 72 herds were positive according to the blood test, but negative according to the ring test. The ring test has thus failed in 72 (13.4%) of the blood-positive herds. That the ring test antigen has been relatively specific is seen from the fact that a report of 243 herds from three dairy districts, including 13 herds which, according to blood test, were infected with brucellosis, showed a 99.6 per cent agreement between the ring test and the blood test.

Seit and Leth Jorgensen believe that they have established proof of the very great specificity of the ring test.

Simultaneously with these results, the author's results were published.[1] As a consequence of the indications by earlier investigators that the tendency to nonspecific ring test reactions was particularly pronounced in the examination of pooled milk while it was less so in the case of samples from individual cows, an evaluation of the former samples was based on incubation for thirty minutes, and of the latter samples for fifty minutes. Readings of the ring tests were, however, made after fifteen, thirty, and fifty minutes' incubation at 37 C., and a fourth and fifth reading after the samples had been left at room temperature for two and twenty hours, respectively, for the determination of the specificity of the test.

By examination of milk samples and blood samples from 587 cows, 123 reactors were demonstrated with the ring test, 97 of which reacted with a blood agglutination titer of

complete or positive at 1:20 or higher, 21 with partial at 1:20, 4 with 1:10 and, finally, 1 with negative titer 1:10. At a blood examination of the last mentioned cow made seven months previously, the cow gave the blood agglutination titer of complete at 1:20, for which reason the last mentioned reaction, too, must presumably be regarded as specific. It should be observed, however, that ring tests performed on colostrum, on pathologically changed secretion, and on "dry secretion" are not included in the above account, partly because it has often been impossible in practice to read the ring test, as there has been no cream-rising capacity, and, also, because reactions occurred which, when correlated with blood tests, had to be interpreted as nonspecific. On the other hand, among the 464 cows which gave a negative ring test there were 24 which reacted with a blood agglutination titer of 1:20 or higher. The results are given in table 1.

By means of the ring test, it was thus possible to detect 97 (80.2%) of the 121 cows which reacted with a blood agglutination titer complete at 1:20 or higher, whereas with this reaction value, the ring test gave a surplus of 26 positive reactions, which is 21.5 per cent of the cows with the titer 1:20 or higher. The disagreement with this reaction value for blood agglutination titer thus comprises altogether 50 cases, 26 of which are surplus ring tests, whereas, conversely, there was a surplus of positive blood tests in 24 cases. True enough, the ring test failed in 3 cases with the blood agglutination titer 1:100 and in 2 cases with the titer 1:200. Later examinations, however, have established that these negative ring test reactions on samples from individual cows which are strong reactors are usually due to lack of cream-rising capacity, for, by mixing these samples with ring-test-negative pooled milk and performing the ring test, positive reactions are obtained — an outcome which at least partly explains why the ring test is more sensitive on pooled milk than on samples from individual cows.

If the lowest reaction value for positive whey agglutination titer is put at + ÷ 1:2.5, the ring test on the previously mentioned material of 587 cows has given seven more positive reactions than the whey agglutination test; however, 15 samples which have reacted positively with the ring test show a negative whey agglutination titer, whereas, conversely, eight samples with positive whey agglutination titers present negative ring tests. The ring test has thus given 1.2 per cent more positive reactions than the whey agglutination test on samples from individual cows.

If comparative examinations of the ring test and the whey agglutination test on herd samples are made, the latter (performed on 109 samples)—always assuming the lowest reaction value to be + ÷ 1:2.5—shows 46 positive reactions, while, after incubation for fifteen minutes, the ring test gives 63 positive reactions; after incubation for thirty minutes, 80; after incubation for fifty minutes, 85; after having been held at room temperature for a further two hours, 92; and after having been left at room temperature for twenty hours following incubation, 93 positive reactions.

It thus appears that the ring test is far more sensitive on herd samples than is the whey agglutination test, whereas this does not hold true of samples from individual cows.

On herd samples, the ring test has already, after incubation for fifteen minutes, given 15.6 per cent more positive reactions than the whey agglutination test; after thirty minutes, 31.2 per cent; after fifty minutes, 35.8 per cent; and after having been left at room temperature for a further two hours, 42.2 per cent. The sensitivity of the ring test thus increases with the time of incubation. A necessary condition for this long incubation time, in order that the ring test antigen employed does not give nonspecific reactions, is that the bacteria count of the milk be comparatively low when the test is started, as the oxidized hematoxylin used in the staining of the Brucella antigen acts as an oxidation-reduction indicator and, therefore, becomes decolorized by vigorous growth of bacteria. That the antigen employed has not given a very great number of nonspecific reactions is seen from the fact that seven samples of pooled milk from a herd which contained neither ring test reactors nor blood test reactors, even at the 1:10 reaction value, were still negative after having been incubated for fifty minutes at 37 C. and thereafter held at room temperature for twenty hours.

The following year, Darnell[3] published an article on the ring test for infectious abortion in cattle, its working conditions and sources of error. It seems warranted to assume, however, that the ring test antigen employed had been nonspecific, since, by examination of milk samples and blood samples from individual cows in two large herds which had been cleared of brucellosis by means of blood tests, Darnell found a number of distinct ring test reactors, not a single one of which presented signs of any agglutinins in the blood, even with the reaction value 1:10.

Darnell, indeed, concludes that, in the hands of inadequately qualified persons, the ring test will be more harmful than bene-

ficial, and he also states that the results obtainable by means of the ring test will be highly dependent on the authority and critical judgment of the individual veterinarian.

It will be understood from this survey of the literature that the value of the ring test as a diagnostic means stands or falls with the higher or lower specificity of the antigen employed.

CURRENT STUDIES

At the instance of the studies undertaken by Seit and Leth Jorgensen and the author, the Danish State Veterinary Directorate decided, in the autumn of 1944, to conduct a large-scale experiment on the value of the ring test as a link in a systematic brucellosis control program. The production of the necessary quantity of ring test antigen was entrusted to the Hygienic-Bacteriological Laboratory of The Royal Veterinary and Agricultural College.

During the work with the production of the ring test antigen to be used in the examinations undertaken by Seit, Leth Jorgensen, and the author, it was proved that the antigen from the different production lots might be of varying quality, depending on the care with which it was produced. The antigen employed in the experiments with milk, which was known for certain to be free of Brucella agglutinins, gave most frequently a bluish violet cream layer which, however, did not present a stronger color than the column of milk below. The margin between a negative and a weakly positive reaction was a very narrow one.

Improvements in Antigen Production.—The question therefore presented itself whether it was possible to produce a ring test antigen which gave a white cream layer with milk samples that were free of Brucella agglutinins, a truly specific ring test antigen. The number of doubtful reactions would in this way be reduced considerably, while at the same time the importance of the subjective estimation would be practically eliminated.

This was accomplished by washing the stained antigen with acidified water (7 cc. 1-N hydrochloric acid in 1,000 cc. tap water) instead of ordinary tap water as originally described by Hermann and, later on, by other authors. For stabilization and preservation, the stained and washed antigen was suspended in glycerin with ½ per cent carbolic acid added, instead of phenolized saline.**

When this objective had been achieved, the next problem presented itself, *viz.*, whether the ring test antigen could be rendered more sensitive. The examinations conducted by Seit and Leth Jorgensen showed that the ring test failed in no less than 13.4 per cent of the herds which blood testing had established as infected with brucellosis.

TABLE 1—Correlation Between the Ring Test and the Slow Agglutination on Blood Samples from 587 Cows from Six Herds with Chronic Brucellosis

	Blood agglutination titer											
	÷1:10	+1:10	p 1:20	t 1:20	1:50	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400
Ring test + / 123	1	4	21	4	21	20	24	10	9	5	2	2
Ring test ÷ / 464	288	107	45	3	16	3	2					

If the ring test was to become generally recognized as a link in the practical work of combating brucellosis, it would be necessary for the ring test antigen to be made so sensitive that in the examination of herd samples it would reveal to the widest extent possible the presence of reactors. This was achieved by intensifying the staining procedure by heating the antigen with the staining solution to 70 C. for five minutes. In this manner, it became possible to dilute the stained Brucella antigen with phenolized glycerin in the ratio 1 Gm. of bacteria to 20 cc. of glycerin, whereas earlier investigators had been diluting it 1:5.

The introduction of these improvements in the antigen production rendered possible extension of the incubation period to one hour without running any risk of nonspecific reactions, even when dealing with pooled milk.

True enough, in previous examinations, the author had demonstrated that the dry secretion, colostrum, and secretion that was greatly changed pathologically, as a rule, exhibit a

**A full description of the procedure employed in the production of specific ring test antigen will be published on completion of the experiments conducted in connection with the production. Patent priority rights reserved.

TABLE 2—Comparative Examinations of Various Ring Test Antigens, Marked I, II, III, and IV

TABLE 2—Comparative Examinations of Various Ring Test Antigens, Marked I, II, III, and IV

Time of incubation at 37C.		To.*	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	Tr.*
15 minutes	I											
	II					+						
	III											
	IV											
30 minutes	I											
	II											
	III											
	IV											
1 hour	I											
	II											
	III											
	IV											

*To. designates milk containing Brucella agglutinins; Tr. designates milk decidedly free of Brucella antibodies.

far higher titer than the blood agglutination titer (up to 100 times higher), and that the same forms of secretions in cows with a negative blood test often give a positive milk agglutination titer. However, as the material was obtained from herds that were chronically infected with brucellosis, and their relationship is inconstant, there is every reason to assume that the agglutinins demonstrated in these categories of secretion should not be regarded as nonspecific but as a local formation of antibodies in the udder produced by foci of Brucella. Subsequent examinations seem to confirm this view.

A general idea of the significance of these improvements in the antigen production is best obtained from the study of table 2, which shows the outcome of comparative examinations of various ring test antigens produced in different, recognized laboratories.

Procedures for Comparison.—The examinations mentioned were carried out by producing a series of increasing dilutions of pooled milk containing Brucella agglutinins in pooled milk that is decidedly free of Brucella antibodies in quantities of 5 cc. Thereafter, 1 cc. of the resultant dilutions was pipetted off and transferred to four rows of test tubes, so that the dilutions found in these corresponded completely to each other. To each row, one of the four ring test antigens was added, after which the samples were mixed and incubated. The evalution of the reactions was made after incubation for fifteen, thirty, and sixty minutes, respectively.

After fifteen minutes incubation, ring test antigen II is most sensitive; ring test antigen IV is least sensitive.

At the evaluation undertaken after incubation for thirty minutes, it appears that ring test antigen II is highly nonspecific, as, even with the pooled milk which is definitely free of Brucella agglutinins, it gives a rather vigorous positive reaction. The greater sensitivity of this ring test antigen after incubation for fifteen minutes is, therefore, only apparent, as even here the reaction must be regarded as a combination of a ring test reaction and a Schern-Gorlisch reaction. If the relative sensitivity of the other ring test antigens is considered, it is seen that ring test antigen IV is decidedly the most sensitive, giving a titer which is four times as high as those of ring test antigens I and III.

After incubation for one hour, ring test antigen II gives the reaction + + +, the most vigorous positive reaction, even with the pooled milk which is definitely free of Brucella agglutinins and has been obtained from a herd which has neither ring test reactors nor blood test reactors, even with the lowest reaction value 1:10. The strongly positive reaction on this milk must be looked upon sim-

ply as a Schern-Gorlisch reaction, which has nothing at all to do with a ring test. In the case of ring test antigens I and III, it seems inadvisable to incubate the samples of pooled milk for one hour before evaluating the reaction, as with both of these antigens the result has been that the cream layer has assumed the same bluish violet color as the milk column below, and, if used in practice, they will no doubt give rise to a relatively great percentage of doubtful reactions. Ring test antigen IV, the antigen produced by the author, even after incubation for one hour, when all the cream must be presumed to have gravitated to the top, is still fully specific, as the cream layer is still white. If, in the case of ring test antigens I and III, the reading (because of possible, nonspecific reactions) is made after only thirty minutes, it will be seen that ring test antigen IV after incubation for one hour gives a titer which is 16 times higher.

Further, it will be seen from the table that when using ring test antigen IV it has been possible to dilute the reacting pooled milk no less than 256 times with pooled milk, free of Brucella agglutinins, and yet retain a positive reaction, or about six times more than Norell and Olson have been able to dilute Brucella-infected pooled milk when using the ring test antigen produced by them. At the same time, this titration illustrates with all possible clarity that in the employment of such a sensitive ring test antigen the very greatest care must be taken to avoid transferring Brucella agglutinins when collecting herd samples.

Comparison of Ring Test and Agglutination Test.—An idea of how much more sensitive the ring test is on pooled milk than is the slow agglutination test is obtained by the study of table 3, in which the ring test antigen has been titrated in the same manner as in table 2, while, at the same time, the milk agglutination test has been performed on the dilutions produced.

It is seen from the table that, in the agglutination test, antibodies have only been traceable in the dilution 1:4, while they have been traceable in the ring test right up to the dilution 1:128. The ring test, in this case, has been about 30 times as sensitive as the agglutination test.

An impression of the behavior of the ring test antigen produced by the Hygienic-Bacteriological Laboratory of the Royal Veterinary and Agricultural College in (field) work with the combating of infectious abortion, is best obtained by the study of M. J. Christiansen's article[2] on the employment of the ring test in combating bovine brucellosis. As the leader in brucellosis eradication in Jutland and Funen, he has published the record of the experience gained in 1945 and 1946 by the use of the ring test on herd samples. The material comprises altogether about 100,000 herds. Of these, about 16,000 herds lie within a specified experimental area comprising 134 dairy districts in which the individual animals in the herds have been blood-tested as a control of the value of the ring test as a diagnostic agent. He also lists 3,952 arbitrarily chosen cows where simultaneous blood and milk tests of the individual animals showed complete agreement in 84.3 per cent, partial disagreement in 4.1 per cent, and total disagreement in 11.6 per cent. No less than 82.3 per cent of the cases which presented complete disagreement showed positive ring test, but negative blood test, indicating that the ring test antigen employed is far more sensitive than any antigen previously described.

DISCUSSION

On basis of the experience gained with the above mentioned material, Christiansen evaluates the ring test as follows:

> A thorough investigation established that the ring test itself only fails entirely in a very small number of cases in herds in which conditions are such that it should give satisfaction. The fact that a herd shows positive ring tests several times in succession, whereas blood tests undertaken in the intervals give negative results, is practically always a consequence of the individual animals, which in these herds cause the reactions in the milk test, giving reactions in the blood test, but generally such a weak blood reaction that it is below the lowest reaction value (agglutination titer 1:20) in use in this country (latent blood reactors).

In the light of this evaluation, one might be led to believe that, in reality, the ring test antigen has been rendered too sensitive. This, however, is hardly the case, as Aage Suurballe[12] observes that, in Bornholm in 1942 to 1945, repeated blood testing of all animals more than 1 year old in 3,800 herds established infection with Brucella in 585 herds which had previously given negative reaction by blood tests. Of these, the animals in no less than 187 herds reacted negatively to blood testing only once, and the animals in 203 herds were negative twice, while previous blood tests had established the presence of the infection in these. If the remaining 195 herds are considered newly infected from without, even though a number are no doubt relapses (cf. later), this group represents 5.1 per cent of the total material examined, whereas the two other groups of 390 herds represent no less than 10.3 per cent. If it is assumed that true new infections in the lat-

ter category are of approximately the same magnitude as in the total material, there is a balance of 370 herds in which there is hardly any new infection from without, but in which the Brucella infection has reappeared because of the constant presence in the herd of the infectious agent in 1 or more of the remaining animals, although to such a negligible extent that it has not been possible to bring up the blood agglutination titer to the lowest reaction value used in this country. The infectious agent, however, has not been quite eradicated. On the contrary, it has had an opportunity of multiplying again, presumably as a result of a reduced resistance in the latently infected animals, combined with an increase in the virulence of the bacteria.

In the light of these considerations, there seems to be every reason to omit registration of herds which have formerly been Brucella-infected, as long as it is possible to demonstrate the presence of infection by serodiagnostic methods, whether it be by blood test or ring test examinations.

In further illustration hereof, an instance can be cited from a herd of about 80 cows which, according to blood testing with the lowest reaction value 1:20, was supposed to have been cleared of infectious abortion on May 1, 1945. Thereafter, the control of brucellosis has been maintained by means of ring test examinations of samples from individual cows, blood samples, however, having been tested from cows whose milk showed a positive ring test reaction.

found which on blood examinations showed a titer below 1:20. Of these, 3 have been removed from the herd so that today, in addition to the cow mentioned previously, there is only 1 old ring test reactor in the herd. At the examination on Jan. 5, 1948, 5 new ring test reactors were, however, in evidence for the first time since the introduction in this herd of the ring test examinations. In all probability, these cases must be ascribed to infection from the above mentioned cow.

Further behavior of the Brucella infection, as a consequence of this flare-up will have to be left open for the present. The activation of the infection may possibly subside without causing further harm to the herd, as the cow in question had calved in a box stall and had no opportunity to transmit the infection when the risk of transmission must be presumed to have been greatest. Still, the question presents itself: What would have happened if the cow had been placed in a herd which had never been in contact with Brucella bacteria, and had been allowed to calve in the row of cows?

Another factor of interest for the diagnosis of infectious abortion is whether the content of antibodies in blood and milk is usually subject to fluctuations depending on the stage in the lactation period, and whether changes, if

TABLE 3—Experiment Illustrating the Relative Sensitivity of the Ring Test and the Agglutination Test on Pooled Milk

	To.**	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	Tr.**
Ring test (inc. 1 hr.)	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	+	(+)	0
Milk agglutination test	+	+ ÷	÷ +							0

**To. designates milk containing Brucella agglutinins; Tr. designates milk free of Brucella antibodies. Inc. = incubation.

In this herd, is a cow born March 1938, blood test and ring test examinations of which demonstrate the interesting features presented in table 4. The cow shows no reaction (since 1:10 is below the lowest reaction value used in this country) from May 1, 1945, to Jan. 20, 1948, when it is a vigorous reactor. The ring test as well as the blood reactions 1:10 show, however, that the cow has not at any time been able to clear itself completely of the infection, but on the contrary, has had a relapse after having been under the minimum reaction value (1:20) for two and one-half years.

In 1945, when ring test examinations of individual cows were introduced in the herd concerned, 5 so-called ring test reactors were

any, in the content of antibodies are parallel in blood and milk. An attempt has been made to throw light on this problem by simultaneous titrations of blood samples and milk samples from 651 cows from six herds chronically infected with abortion. The material has been divided into five periods according to the time which elapsed since the last calving The average titer for the milk samples as well as the blood samples and the relation between these have been calculated for the various groups. The results are presented in table 5.

If the average blood titer is first considered, it is seen to be lowest in the first period, which is in harmony with the fact that the cows usually clear themselves of

their uterine infection shortly after calving. Thereupon the average blood titer rises during the second and third periods, reaching its peak in the fourth period, presumably because the massivity of the infection and, consequently, the formation of antibodies is increased with advancing pregnancy. Finally, the interesting phenomenon is observed that the average blood titer declines very considerably in the fifth period. This position must no doubt be ascribed to the fact that the cows belonging to this group have either not become pregnant at all or have become so only at a relatively late stage in the period and may thus be taken to constitute a confirmation of the fact that nonpregnant cows clear themselves of the uterine infection far better than do pregnant cows.

A study of the relation between the average milk titer and the average blood titer shows a clear variation from period to period. The ratio is smallest in the second period. Incidentally, the ratio is inversely proportional to the amount of secretion if the first period is omitted. When the ratio is greater here than in the second period, it must be ascribed to the fact that the material in group 1 includes some cows that have just calved, and the colostrum is excessively rich in antibodies as compared with the content of antibodies of the blood. The cows coming under group 5 have seemingly been able to clear themselves far better of their Brucella infection, not only of the uterus, but also of the udder, than have the cows in group 4.

TABLE 4—Examination of a Cow's Reactions with Blood Tests and Ring Tests

Date of calving	Ring test reaction	Test date	Blood test titer	Test date
			1:20	12/20/42
1/ 5/43			1:20	2/13/43
			1:20	4/ 2/43
			1:20	7/ 5/43
			1:20	10/23/43
			1:20	12/ 9/43
			1:50	3/ 2/44
4/ 8/44			<1:20	5/ 5/44
			<1:20	6/ 1/44
			<1:20	7/ 2/44
			1:50	10/10/44
3/ 7/45			p 1:10	5/ 1/45
	+ + +	11/10/45	1:10	11/20/45
1/20/46	+ +	3/ 6/46	p 1:10	3/15/46
	+ + +	9/20/46	1:10	10/ 5/46
12/12/46				
11/18/47	+ +	1/ 5/48	1:100	1/20/48

The relationship between average milk whey titers and average blood titers fluctuates considerably within the lactation period; the average milk titer even exceeds the average blood titer in the fifth period. This seems to suggest that a significant part of the antibodies secreted with the milk is formed in the Brucella-infected udders. At the same time, the preceding examinations give the interesting information that, if performed on pooled milk obtained principally from cows at a late stage in the lactation period, the ring test gives a positive reaction more often than if performed on pooled milk from cows which are at their highest yield. Gradually, as the practice of making the cows calve in the spring is more widely adopted, the ring test, will therefore, reveal more heards as infected with Brucella in the autumn and winter than in the spring and summer.

SUMMARY

On the basis of a critical review of the literature on the *Brucella abortus* ring test, it has been established that the ring test antigen previously described has been nonspecific and as such has given rise to nonspecific, positive reactions.

It has been demonstrated that the ring test antigen produced by the author, and employed in practice in the combating of infectious abortion, is specific and far more sensitive than any ring test antigen previously described.

Attention is called to the serious problem which relapse cases constitute in herds which have been cleared of the infection on basis of blood tests.

An attempt has been made to throw light on the fluctuations in the content of Brucella antibodies in milk as well as in blood, and the relative proportion between Brucella antibodies in these during the lactation

TABLE 5—Comparative Examinations of the Content of Antibodies in Blood and Milk, Illustrated by Simultaneous Titrations of Milk Samples and Blood Samples from 651 Cows

Group	1	2	3	4	5
Months after last calving	0-3	3-6	6-9	9-12	>12
No. of cows	161	155	119	145	71
Ave. whey titer	1:15.3	1:10.5	1:32.2	1:350	1:141
Ave. blood titer	1:101	1:107	1:145	1:821	1:108
Ratio milk titer to blood titer (ave.)	1:6.6	1:10	1:4.5	1:2.3	1:0.77

period, by means of titrations performed simultaneously on milk and blood samples from 651 cows.

It appears that, provided the antigen employed is specific and sufficiently sensitive, the *Brucella abortus* ring test has given us a quick, easy, and reliable test for diagnosing brucellosis in the herds. The experience gained to date with the practical application of the test indicates that with ring test examinations of herd samples, performed at relatively short intervals, it is possible to map the herds infected with Brucella in a far more reliable and easy manner than with blood testing once a year. This factor is important in (field) work in the combating of brucellosis, as by this means a possibility has been opened up of checking, in a far more efficient and rational manner, that thorn in the flesh of collective brucellosis control: the newly infected herds. To this should be added that, as a rule, the herds will not be declared clear at such an early stage on basis of the ring tests as on basis of the blood tests, which seems particularly expedient for the purpose of avoiding relapse cases.

Gradually, as the eradication of brucellosis proceeds on an area basis, it is possible that future examinations will make the use of an even more sensitive ring test antigen necessary in order to remove the remaining cases of the latent infections. At the same time, the lowest reaction value in blood test examinations will probably have to be put down from 1:20 to 1:10. As to the sensitivity of ring test antigen, preliminary investigations show that it is possible to produce an antigen which is twice as sensitive as that hitherto employed in field work.

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Sodium Fluoroacetate (Compound 1080) Poisoning in Sheep

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SODIUM fluoroacetate was found to be a most effective mammal control agent through studies conducted during the period of July, 1944, to August, 1945, when its potential utility was announced.[1] Early in the field use of this new poison for the control of ground-burrowing rodents, it became apparent that compound 1080 possessed toxicity characteristics which would endanger beneficial birds and animals, and a number of articles were released warning against careless handling of baits carrying sodium fluoroacetate.[2-7] At the same time, investigations were started to evaluate the actual hazards to many forms of life inherent in 1080, and publications dealing with the pharmacology of the poison,[8] its clinical effects on horses and dogs,[9] and its lethal doses on a large number of different birds and animals appeared.[10]

The unique danger to range animals, such as sheep and cattle, which might be herded over areas which had been treated with 1080-poisoned grain for prairie dog or ground squirrel control, was recognized. Operational crews were warned to notify users of the land before any bait was distributed, and regulations were set up to limit the handling of the poisoned baits to trained personnel. It was inevitable, however, that the poison would be blamed for unexplained stock losses in areas where it had been used. Unfortunately, there is no chemical method for positive identification of sodium fluoroacetate in the tissues of animals poisoned with this substance. The present investigation was undertaken, therefore, to determine the actual lethal dose of 1080 for sheep, and to learn whether the symptomatology or the pathologic findings would be diagnostic. An additional aim of the experiment was to observe the palatability of the standard field rodent poison to sheep and to evaluate organic changes in those animals picking up enough of the grain from bait spots to cause death.

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MATERIALS AND METHODS

Sheep.—Twelve healthy animals were used. These were between 9 and 12 months old, with one exception which was a female 8 years old. Both sexes were included. Prior to the experiment, the animals were maintained in a single pen and fed alfalfa hay and a small quantity of oats daily for a period of several weeks. At the time the experiment began, their physical condition was medium. Each animal was weighed separately, and feed was withheld for twenty-four hours immediately prior to administration of the compound. Two sheep were used for each level, as shown in table 1. For each sheep, two hemograms were made prior to the experiment, and two each after the experiment for those that survived. Dead animals were autopsied within one hour after death. Heart, brain, stomach, intestine, liver, and kidney were fixed in 10 per cent neutral formalin, sectioned, and stained with hematoxylin and eosin.

Pens.—While the animals were being fed the compound, they were maintained in separate, small pens. Immediately following administration, they were placed together in a large indoor pen with clean water and alfalfa hay available.

Sodium Fluoroacetate.—It was necessary to use two different mixtures because some animals refused to consume voluntarily, assigned doses in the form of a modified field rodent bait. The first mixture duplicated the field rodent formula of the United States Fish and Wildlife Service, with the exception that the concentration of the poison was modified so that each gram of bait contained 1 mg. of sodium fluoroacetate. It was prepared by dissolving the poison in water and emulsifying the solution with a blend of mineral oil, soybean oil, and lecithin. The emulsion was mixed evenly with whole oats.

The second mixture was composed of finely ground oats, sodium fluoroacetate, and sufficient water to provide a pasty consistency. It could be forced through a stomach tube with ease. One cubic centimeter of this mixture

TABLE I—Sodium Fluoroacetate Poisoning in Sheep

TABLE I—Sodium Fluoroacetate Poisoning in Sheep

Sheep No.	Sex	Body wt. kg.	Mg. of 1080/kg. bd. wt.	Mg. of 1080/Gm. of grain	Total dose of grain, Gm.	Administration		Time of first symptoms	Time of death	Hr. from administrat. to death	Hr. of sickness
						Route	Time				
11	F	29.5	0.25	1	7.37	F*	1:30 p.m.	none	survived		
5	F	23.6	0.25	1	5.90	ff**	3:45	none	survived		
17	M	21.8	0.50	1	10.90	F	1:30	9:30 p.m.	11:05 p.m.	9.50	1.50
13	M	34.1	0.50	1	17.05	ff	3:35	6:30 a.m	8:15 a.m.	16.25	1.75
8	F	22.3	0.75	1	16.75	ff	3:45	11:00 p.m.	11:45 p.m.	8.0	0.75
14	F	30.9	0.75	1	23.17	F	1:30	10:20 p.m.	10:45 p.m.	9.25	0.50
6	F	14.5	1.00	1	14.50	ff	3:45	1:00 a.m.	2:00 a.m.	10.25	1.00
10	M	40.9	1.00	1	40.90	F	1:30	7:00 p.m.	7:15 p.m.	5.75	0.25
7	F	23.6	2.50	1	59.00	ff	3:45	7:30 p.m.	8:15 p.m.	4.5	0.75
3	F	24.5	2.50	1	61.25	ff	3:45	7:30 p.m.	8:30 p.m.	4.75	1.00
15	M	19.5	10.00	1	195.00	ff	3:45	6:00 p.m.	6:30 p.m.	2.75	0.50
18	F	43.2	10.00	1	432.00	ff	3:45	6:00 p.m.	6:30 p.m.	2.75	0.50

*Fed voluntarily.
**Force-fed.

contained 1 mg. of sodium fluoroacetate. Both mixtures were fed while fresh.

Administration.—Dosage of the drug was based on body weight. Six levels were given as follows: 0.25, 0.50, 0.75, 1.00, 2.50 and 10.00 mg. per kilogram of body weight. Two sheep were used for each level. At the beginning of the experiment, each animal was offered its dose in the form of the special field rodent bait to be eaten voluntarily. After fifteen minutes, the uneaten portion of each dose was force-fed in the form of grain-drug-water paste by means of a stomach tube. Thus, each animal received its total dose within a relatively short period of time. Continuous observations were made from time of administration until death or disappearance of symptoms.

RESULTS

Palatability.—The modified field rodent bait seemed unpalatable. Each animal ate only small quantities voluntarily. Sheep receiving the smallest doses, however, consumed the entire amount voluntarily, as shown in table 1.

Symptoms.—The time interval from administration until manifestation of symptoms varied inversely with the size of the dose (table 1). The duration of symptoms varied from one-half to one and three-fourths hours. All animals that showed symptoms died. The onset was characterized by convulsive contractions of groups of muscles. Those of the back were involved commonly and from them, the spasms spread to involve all muscles. Uneasiness was pronounced with frequent changing from standing to reclining position. In the early stages, the animals showed a preference for the rump-sitting posture, as if to relieve pressure on the heart from the diaphragm. As weakness developed, the reclining position was assumed. Periods of rest were interrupted by periods of excitation in which the affected animal ran blindly about the pen, colliding with any obstacle in the path of movement. In the terminal stages, animals ran violently about the pen, collapsed, and died in convulsions.

Heart rate increased, as was determined by means of a stethoscope. Four hours after administration, heart rate varied from 120 to above 400, averaging 218 per minute in 9 of the poisoned animals, while

3 normal animals varied from 98 to 120, averaging 112 per minute. Toward the terminal stages, the pulse became fast and weak. In some cases, it was not perceptible. In all sheep that died, heart action ceased before respiration. All animals died except the pair on the lowest level of the compound.

Autopsy.—Few significant changes were observed. The heart contained multiple subepicardial petechiae. The atrioventricular valves contained a few hemorrhages. Heart action had stopped in a state of diastole. The lungs contained some extravasated blood, which in some cases was mixed with frothy transudate in the bronchi and trachea. Other organs were normal.

Histopathology.—No consistent histologic changes were observed in the organs studied.

In the two animals that survived the experiment, hemograms prepared at intervals up to six weeks after the experiment showed no significant change from hemograms made prior to the experiment.

DISCUSSION

Even though sodium fluoroacetate seems to be of low palatibility, it may cause accidental poisoning and economic loss by virtue of the extremely low minimal lethal dose. It is suggested that 1080 be used with caution in the control of noxious mammals, and that sheep, and probably cattle, be excluded from areas where it is exposed.

From observations made during the course of the experiment, it appears that the drug causes death by alteration of heart function.

SUMMARY

1) Under conditions of the experiment, the m.l.d. of sodium fluoroacetate for sheep was between 0.25 and 0.50 mg. per kilogram of body weight. The m.l.d. was also the minimal dose producing symptoms.

2) The time interval between administration and manifestation of symptoms varied inversely with the size of the dose.

3) Symptoms of poisoning were motor irritation, excitation, rapid pulse becoming weak, general weakness, convulsions, and death; heart action ceased before respiration. All animals showing symptoms died. The 2 survivors received the lowest dosage and appeared normal at all times.

4) A modified field rodent bait prepared with steam-rolled oats, containing 1 mg. of sodium fluoroacetate per gram of grain was of low palatability for the sheep used in this experiment.

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DDT Emulsions for the Destruction of Lice on Cattle, Sheep, and Goats

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LICE ARE among the most important and widespread external parasites of livestock in the United States. Although losses attributable to these pests have not been ascertained, they add up to a significant figure. Lice are more injurious to cattle than to other animals. In farming communities, poorly housed cattle frequently suffer from intense skin irritation as a result of louse infestations. Range cattle are less often parasitized than dairy and feeder cattle, and heavy infestations are usually found only on a few susceptible hosts in each herd. In grossly infested animals, however, the lowered vitality during unfavorable weather may contribute to the death of an occasional animal.

In sheep, lice not only irritate the skin and rob the host of nutriment, but the chewing lice damage the wool fibers, and both chewing and sucking lice soil the fleece. Goats are known to harbor at least five different kinds of lice with resultant loss of vitality and, in Angora goats, marked reduction in mohair clip.

For many years, a number of chemical compounds have been widely used for the destruction of lice. In the past, the most successful and the most commonly employed parasiticides have been nicotine sulfate, various coal tar derivatives, arsenic compounds, and rotenone-containing materials. Many thousands of farm and range animals throughout the nation are still being treated with preparations containing these materials. On the whole, the afore-mentioned products have given good results in controlling louse infestations. Rotenone-containing products, in particular, such as derris and cube powders, have provided a relatively safe, inexpensive, and effective means of destroying lice. Nicotine sulfate and arsenic, however, are toxic materials, and the use of specially designed field tests are required to determine the concentration of the chemicals in the dipping vat. Even the coal tar products have proved dangerous at times to farm animals, especially when used in high concentration or when the contents of dipping vats were not thoroughly stirred.

Aside from the toxicity of some of these mixtures used extensively in the past, the chief drawback has been the need to treat the animals repeatedly in an effort to eradicate louse infestations. None of the previously named agents are ovicidal, nor do they have any residual effect upon the lice which continue to hatch from eggs remaining upon the animals after motile lice have been destroyed. Ranchers and farmers, especially the former, find it entirely impractical to assemble their livestock for two, three, or even more treatments at intervals of from fifteen to twenty days, in order to achieve effective control of lice. Their urgent need, therefore, is for a material which will eradicate the pests with a single application. The introduction of DDT and the information which soon became available regarding its residual properties aroused the hope that a single treatment with this chemical might be sufficient to destroy all motile lice, and either destroy their eggs or the newly hatched lice as they issue from the eggs.

The investigations described in this paper include pen and field tests in which emulsions containing DDT were applied to cattle in spray and dip form, and to sheep and goats in the form of washes, dips, and wading troughs.

MATERIALS AND METHODS

Tests with DDT Sprays for the Destruction of Lice on Cattle.—The test animals were grade, range Herefords, selected because they

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were grossly infested with the short-nosed cattle louse, *Hematopinus eurysternus*. Five animals were confined in pens at Las Vegas, and 2 at Albuquerque, N. M. Six experiments were conducted during the spring and fall months in 1945. The test animals were maintained in pens from one to three weeks before the start of each experiment, during which period, it was observed that the degree of infestation was not reduced by the change of environment.

The DDT-oil-water emulsions, as a rule, were prepared by first dissolving DDT in technical grade benzol, either at room temperature or, to hasten solution, at 120 F. Oils containing emulsifiers were then added to DDT solution. The two most satisfactory products for this purpose were found to be (1) emulsifiable petroleum oil (soluble oil-D—Texaco) of a variety commonly used in both industry and agriculture, and (2) saponified pine oil. To these solutions, equal quantities of water at approximately 140 F. were added with constant stirring. The resulting, creamy emulsions were relatively stable and were held as stock emulsions until ready for dilution with cold water in spray tanks, immediately prior to use. In all tests but one, the finished emulsions contained 0.25 per cent DDT. In test 3, the single exception, the emulsion contained 0.34 per cent DDT.

The emulsions were applied to the test cattle by means of hand-operated, barrel-type sprayers, at approximately 50 lb. nozzle pressure. Not less than 10 gal. of fluid were applied to each animal, to assure complete wetting of the skin and saturation of the hair. Examinations of treated animals were made daily for seven days following treatment and thereafter at frequent, irregular intervals. Only those observations made on the seventh, fourteenth, thirtieth, and sixtieth days after the application of the parasiticides are herein recorded. The test animals were isolated from other cattle during these periods.

As shown in table 1, in tests 1, 2, 3, 5, and 6, a single application of DDT emulsions in spray form was made. In test 4-4a, the cattle were sprayed a second time (test 4a, table 1) three weeks after the first treatment, in order to determine the relative effectiveness of two DDT applications for the eradication of cattle lice.

Tests with DDT Dips for the Destruction of Cattle Lice.—The 2,441 cattle employed in this series of tests were taken from herds of range Herefords grazing in central New Mexico. The work was performed during the

TABLE 1—Tests with DDT Sprays for the Destruction of Cattle Lice

Test No.	Date (1945)	Percentage of ingredients in sprays: Ingredients	Percentage	Animals sprayed, (No.)	Species of lice	Comparative No. of live lice, specified days after treatment: 7	14	30	60
1	2/27	DDT Benzol Emulsifiable petroleum oil Water	0.25 0.50 0 75 98.50	1	*H. eurysternus*	F.	F.	F.	F.
2	3/9	DDT Benzol Emulsifiable petroleum oil Water	0.25 0.50 1.00 98.25	1	*H. eurysternus*	F.	F.	F.	F.
3	3/13	DDT Benzol Emulsifiable petroleum oil Water	0.34 0.68 1.02 97.96	2	*H. eurysternus* *L. vituli*	F. F.	F. F.	F. F.	F. F.
4	4/28	DDT Benzol Emulsifiable petroleum oil Water	0.25 0.50 0.75 98.50	1	*H. eurysternus*	F.	F.	*	*
4-a	5/19	Same as test No. 4		1	*H. eurysternus*	N.	N.	N.	N.
5	10/4	DDT Benzol Saponified pine oil Water	0.25 0.25 1.25 98.25	1	*H. eurysternus* *B. bovis*	N. N.	N. N.	N. N.	N. N.
6	10/16	DDT Benzol Saponified pine oil Water	0.25 0.50 0.75 98.50	1	*H. eurysternus*	N.	F.	F.	F.

*A few adult lice were found on the twenty-first day after treatment. Animals re-treated that day and designated in the table under test 4-a.
F. = few; N. = none.

fall of 1945 and the spring of 1946. The cattle involved in tests 1, 2, 4, and 5 constituted entire range herds, some members of which were grossly infested with lice, while others harbored no lice, as far as could be determined. The short-nosed cattle louse, *H. eurysternus*, was found in very large numbers on from 1 to 5 per cent of the animals in each of these herds and in lesser numbers on approximately 10 per cent more. The long-nosed or calf louse, *Linognathus vituli*, was usually confined to the calves and yearlings. The common red or biting louse, *Bovicola bovis*, was found in small numbers on a few animals in all range herds.

The herds were all dipped in vats on, or adjacent to, their home ranches. After dipping, the animals were pastured under fence on the range. The heavily infested animals in each herd were paint-branded before dipping and were examined at frequent intervals for sixty days after treatment.

For test 1, the dipping vat was freshly charged. The large herd in test 2 was dipped in the same vat one day later, after the original dip level was reëstablished by the addition of a small quantity of fresh materials. In the same manner, the herd in test 4 was dipped in a fresh emulsion, and herd 5 followed through the same vat, after replenishing the next day. In performing test 5, however, the concentration of DDT in the vat was increased by 0.03 per cent over that employed in test 4.

For test 3-3a, 6 animals, all heavily infested, were selected from several herds of range Herefords. The subjects, harboring only the short-nosed louse, were dipped in a small experimental vat and were held in isolation in an adjacent corral for three weeks. At the end of this period, the vat contents were brought up to proper level by the addition of a small amount of fresh materials, and the 6 test cattle were dipped once more. The cattle were returned to the corral where they were isolated for two months longer. All 6 animals were examined at least twice weekly following both treatments.

As shown in table 2, in tests 1, 2, 3, and 3a, emulsions containing 0.25 per cent DDT were used. Test 4 involved the use of 0.3 per cent DDT and in test 5, 0.33 per cent DDT was used. The emulsions in the dipping vats were substantially those employed in the spray tests described earlier in this paper, with the exception that xylol was used in place of the more toxic benzol as a DDT solvent in four of the six experiments.

Tests with DDT for the Destruction of Lice on Sheep and Goats.—The animals involved in these experiments were grade farm and range Rambouillet sheep, grade range goats of nondescript breeding, and purebred Toggenburg milk goats. The 11 sheep were moderately infested with the foot louse, *Linognathus pedalis*, and the 6 goats with both the common sucking and biting body lice, *Linognathus stenopsis* and *Trichodectes climax*. The ex-

TABLE 2—Tests with DDT Dips for the Destruction of Cattle Lice

Test No.	Date	Percentage of ingredients in dips: Ingredients	Percentage	Animals dipped (No.)	Animals examined (No.)	Species of lice	Comparative No. of live lice, specified days after treatment: 7	14	30	60
1	10/28/45	DDT Xylol Saponified pine oil Water	0.25 0.25 1.25 98.25	570	6	*H. eurysternus* *L. vituli* *B. bovis*	...	N. N. N.	N. N. N.	N. N. N.
2**	10/29/45	Same as above		830	30	*H. eurysternus* *L. vituli*	...	...	F. N.	...
3	12/ 7/45	DDT Benzol Emulsifiable petroleum oil Water	0.25 0.50 0.75 98.50	6	6	*H. eurysternus*	F.	F.	*	*
3a†	12/29/45	Same as above		6	6	*H. eurysternus*	N.	N.	N.	N.
4	3/ 6/46	DDT Xylol Saponified pine oil Water	0.30 0.30 1.50 97.90	489	12	*H. eurysternus* *L. vituli* *B. bovis*	...	N. N. N.	...	N. N. N.
5‡	3/ 7/46	DDT Xylol Saponified pine oil Water	0.33 0.66 1.65 97.36	540	6	*L. vituli* *B. bovis* *H. eurysternus*	...	N. N. N.	...	N. N. N.

*A few adult lice were found on twenty-first day after treatment. Animals re-treated that day and designated in table under test 3-a.
**Vat contents of test 1 replenished and used.
†Vat contents replenished and used for second dipping twenty-first day.
‡Vat contents of test 4 replenished to higher concentration.
F. = few; N. = none.

periments were performed at Las Vegas and Albuquerque, N. M., during the winter of 1944 and the spring and summer of 1945. As in the case of the experiments involving the use of DDT sprays on cattle, the sheep and goats were kept in individual laboratory pens from one to three weeks prior to the start of each test, and for at least sixty days after treatment.

In tests 3, 4, 5, and 6, the DDT-oil-water emulsions were prepared in precisely the same manner as those used in the cattle louse experiments. Tests 1 and 2, however, preceded all attempts to include DDT in aqueous emulsions, and the products employed in these experiments were rather crude combinations of DDT, a solvent, kerosene, and an emulsifier. The emulsions used in the six experiments contained from 0.1 to 1 per cent DDT. The animals were treated in small dipping vats in four of the six tests. In test 1, the sheep were held in a standing position in a trough containing 10 in. of dip so that only their feet and legs were soaked. In test 2, the goats involved were sponged with the emulsion.

EXPERIMENTAL DATA.

The experimental data are given in tables 1, 2, and 3, which show the percentages of the various ingredients in the emulsions, the method of application, the number of animals treated, the comparative number of live lice at specified intervals after treatment, and other pertinent information. The date on which each experiment was performed is also included, since seasonal variations and atmospheric conditions are known to exert important influences on louse numbers.

An important result not tabulated is the observation that all motile lice were destroyed within twenty-four hours by each of the emulsions used.

In table 1, test 5, a single application of an emulsion containing 0.25 per cent of DDT, applied as a spray, eradicated infestations of short-nosed and red biting lice from 1 test animal. In five comparable tests (1, 2, 3, 4, and 6), however, single applications of emulsions containing from 0.25 per cent to 0.34 per cent DDT failed to eradicate infestations of short-nosed or long-nosed lice. Two sprayings of a test animal, twenty-one days apart, with an emulsion containing 0.25 per cent DDT, as shown in test 4-a, resulted in eradication of an infestation with short-nosed lice.

Table 2 shows that DDT emulsions used as cattle dips were generally successful in eradicating louse infestations. In tests 1, 4, and 5,

TABLE 3—Tests with DDT Emulsions for the Destruction of Lice on Sheep and Goats

Test No.	Date	Percentage of ingredients in emulsions: Ingredients	Percentage	Method of application	Class and No. of animals	Species of lice	Comparative No. of live lice, specified days after treatment: 7	14	30	60
1	11/28/44	DDT	0.25	Wading trough	Sheep 8	*L. pedalis*	N.	N.	N.	F.
		Methyl acetone	1.25							
		Kerosene	1.75							
		Sodium lauryl sulfate	0.15							
		Water	96.60							
2	1/19/45	DDT	0.10	Sponge	Goats 2	*L. stenopsis*	N.	N.	N.	N.
		Benzol	0.30							
		Kerosene	1.00							
		Soap	0.20							
		Water	98.40							
3	4/21/45	DDT	0.25	Dip	Sheep 1	*L. pedalis*	N.	N.	F.	...
		Benzol	0.50							
		Emulsifiable petroleum oil	1.00							
		Water	98.25							
4	4/21/45	DDT	0.50	Dip	Sheep 1	*L. pedalis*	N.	N.	N.	...
		Benzol	1.00							
		Emulsifiable petroleum oil	2.00							
		Water	96.50							
5	4/21/45	DDT	1.00	Dip	Sheep 1	*L. pedalis*	N.	N.	N.	...
		Benzol	2.00							
		Emulsifiable petroleum oil	4.00							
		Water	93.00							
6	5/26/45	DDT	0.10	Dip	Goats 4	*L. stenopsis*	N.	N.	N.	...
		Benzol	0.20			*T. climax*	N.	N.	N.	
		Emulsifiable petroleum oil	0.30							
		Water	99.40							

N. = none; F. = few.

in which cattle were dipped once in emulsions containing from 0.25 to 0.33 per cent DDT, eradication of infestation with short-nosed, long-nosed, and red biting lice was apparently accomplished. Test 2 shows, moreover, that following the passage of large numbers of cattle through the emulsion employed in test 1, the DDT that remained in the dip continued to be completely effective against long-nosed, but not against short-nosed, lice. Test 3-3a shows that, while a single dipping in an emulsion containing 0.25 per cent DDT failed to eradicate infestations of short-nosed lice, a second dipping three weeks later resulted in total eradication.

Single applications of dips containing 0.25 per cent DDT, as shown in table 3, failed to eradicate infestations of foot lice from sheep, (tests 1 and 3). In two additional tests (4 and 5), one dipping in emulsions containing 0.5 and 1 per cent DDT eradicated such infestations. A single application, by means of a sponge, of an emulsion containing only 0.1 per cent DDT eradicated an infestation of common biting lice from goats (test 2). In a similar test (6), a dip containing 0.1 per cent DDT eradicated infestations of both common biting lice and blue sucking lice from goats.

DISCUSSION

In the performance of the work herein outline, a total of 2,449 cattle, 11 sheep, and 6 goats were treated with DDT-oil-water emulsions of various kinds. When dipped, the animals remained in the dip for fifteen to twenty seconds and, in all cases, heads were submerged momentarily. Sprayed cattle received at least 10 gal. of emulsion per head per treatment. Many of the cattle were dipped during relatively cold, windy weather and, in other cases, the cattle were exposed, after treatment, to the hot sun. Some of the sheep were treated when in full fleece, and the wool adjacent to the skin remained wet for as long as forty-eight hours. It is exceedingly gratifying, therefore, to note that under no circumstances was injury to a test animal observed. Naturally, the indiscriminate application of parasiticidal emulsions to farm and range livestock cannot be recommended solely on the basis of such observations. Farm and range practice may occasionally suggest the repeated use of DDT emulsions, but the possible injurious effect of such treatment upon animals has not yet been evaluated.

DDT emulsions were not applied during the months of June through September, inclusive, a period of normally marked reduction in the louse infestations. There is, therefore, no evidence to the effect that the emulsions used are not harmful under the influence of extremely hot weather or prolonged exposure to sunlight. Nevertheless, there is sufficient evidence presented in this paper to show that these same oil emulsions containing 1.0 per cent or less DDT, applied as sprays or dips during the fall, winter, and spring in the Southwest are apparently harmless to cattle, sheep, and goats.

For experimental purposes, the choice between dipping and spraying of livestock for eradication of louse infestation is largely determined by the facilities available at the place of treatment, and the numbers of animals to be subjected to treatment. It is obviously expensive to prepare dip for 1 or 2 head of cattle. Therefore, in performing pen tests on individual animals, hand-operated spraying equipment was used. Experimental hosts treated in this manner, however, were sprayed with excessive quantities of fluid, and were wetted quite as thoroughly as would have been the case had they been dipped. However, in practical farm and ranch operations, where many cattle are to be sprayed, the use of such excessive quantities is not feasible, and very few animals would receive such thorough wetting. Where a dipping vat is available, and where the number of animals to be treated warrants filling the vat, it is certainly advantageous to resort to dipping. It is the opinion of the writers that no other method of application serves to wet the entire body surface as does complete submersion of the animal.

As mentioned earlier in this paper, the short-nosed cattle louse was found in large numbers on the test animals and the long-nosed or calf louse in lesser numbers. The red louse numbers were very small. At examinations made subsequent to treatment, the short-nosed cattle louse was found most frequently, and the long-nosed louse occasionally, but the red louse was never seen. The inference is that infestations of the

red louse were eradicated by single applications of all the emulsions tested. Because it is extremely difficult to find a few lice on the huge body surface of mature cattle, it will be necessary to postpone drawing final conclusions until tests are made in which heavy infestations of the red louse are involved.

Single applications of dilute DDT-oil-water emulsions to cattle eradicated short-nosed and long-nosed louse infestations in some instances whereas, in others, live lice were found as early as seven days after treatment. Precisely why the results were inconstant is not known. The fact remains that one application of emulsions containing as much as 0.34 per cent DDT cannot always be depended upon to eradicate infestations of the short-nosed and the long-nosed cattle louse. In two instances (table 1, test 4-4a, table 2, test 3-3a) in which the first application of the parasiticides failed to eradicate infestations of the short-nosed louse, a second treatment, three weeks later, accomplished this objective.

SUMMARY AND CONCLUSIONS

1) Single applications of DDT-oil-water emulsions containing either 0.25 or 0.34 per cent DDT, applied to cattle by means of hand-operated spraying equipment, eradicated infestations with lice of the species *Hematopinus eurysternus* in one instance but failed to do so in five comparable tests.

2) Single dippings of cattle in DDT-oil-water emulsions containing from 0.25 to 0.33 per cent DDT, eradicated infestations with lice of the species *H. eurysternus* in three tests but failed to do so in two other comparable tests. However, this treatment eradicated, without exception, infestations with lice of the species *Linognathus vituli* and *Bovicola bovis*.

3) Two treatments of cattle with DDT-oil-water emulsions containing 0.25 per cent DDT in both spray and dip form, with an interval of three weeks between treatments, eradicated infestations of *H. eurysternus*.

4) Single treatments, either by dipping or hand application, with DDT-oil-water emulsions containing as little as 0.1 per cent DDT, successfully eradicated infestations of *Linognathus stenopsis* and *Trichodectes climax* from goats.

5) One dipping in DDT-oil-water emulsions, containing 0.5 per cent DDT, eradicated infestations of *Linognathus pedalis* from sheep. Similar treatment with emulsions containing 0.25 per cent DDT destroyed all motile lice, but failed to kill the young lice which issued from eggs attached to the hair and wool on the legs.

6) Evidence is presented which indicates that single applications of emulsions containing 0.25 per cent or more of DDT eradicated light infestations of *Bovicola bovis* from cattle.

7) Applications of DDT-oil-water emulsions, containing from 0.1 to 1 per cent DDT, caused no discernible injury to 2,449 cattle, 11 sheep, and 6 goats.

The Status of the Sodium Fluoride Treatment for Removal of Large Roundworms from Swine

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A TREATMENT for the removal of large roundworms (*Ascaris lumbricoides*) from swine was described in 1945.[10] As developed experimentally and generally employed in practice, it consists in the administration of sodium fluoride in dry, ground feed at a concentration of 1 per cent for one day. Because much has been learned about this treatment from both experimentation and field use, it is desirable to take inventory of the established facts, adding new data on some aspects of the medication.

The Origin of the Sodium Fluoride Treatment

The treatment was developed by workers of the U. S. Bureau of Animal Industry specifically to meet the need for a suitable anthelmintic for swine. Because ascarids are the largest, most common, and most injurious worm parasites of swine, efficacy against them is essential in any anthelmintic of general use in this class of animals. The inadequacy of the older anthelmintics, chiefly oil of chenopodium and phenothiazine, which also were introduced as swine ascaricides by the Bureau,[8] was recognized at least as early as 1942.[14] Between 1942 and 1945, trials against swine ascarids were carried out with some 134 compounds. Under the circumstances tested, sodium fluoride was the most promising. A comprehensive analysis of the three treatments under the discussion was made in 1945.[5]

Literature

The pertinent information on the sodium fluoride treatment is contained in some 20 publications (cited, *vide infra*), although announcements, releases, and abstracts have been numerous. Moreover, much of the relevant literature, including a patent,[7] is of historical or other interest but does not contribute to a knowledge of the performance of the medication. All information on the efficacy and safety of sodium fluoride as a swine ascaricide is covered in seven articles.[1,2,3,10,11,15,17*] In the data reported therein, 598 pigs were used in trials with sodium fluoride, of which 572 were given the chemical in therapeutic, toxicologic, or clinical trials, while 26 were retained, for one reason or another, as untreated controls. In therapeutic trials, concentrations in feed from 0.3 to 2 per cent for periods from one to three days were tested. In toxicity trials, dose rates up to 5 per cent in feed and 1 Gm. per pound of body weight were tested.

Specific Considerations

Efficacy.—In determinations of the efficacy of medicated feeds, data are available on 319 pigs, 75 of which were treated individually, 60 in pairs, and 184 in groups of 2† to 30 pigs each. The treatment removed 95 per cent (2,264) of 2,369 ascarids from 308 pigs. It also removed 93 per cent (3,631) of 3,903 stomach worms from 43 pigs, 30 per cent (2,422) of 8,020 nodular worms from 53 pigs, 20 per cent (302) of 1,493 whipworms from 37 pigs, and none of 25 thorn-headed worms from 6 pigs (table 1, A to E). Obviously, the test pigs were selected because they had ascarids and, in the majority of instances, attention was given only to action against these worms. Regarding other species, however, the data are ample to establish the limited action of the drug against them. The removal of stomach worms at a dosage of 1 per cent in feed is not as high (62%) as indicated by the overall data. The drug is ineffective against nodular worms, whipworms, and thorn-headed worms. It is of interest to note, however, that the highest dosage employed by Roberts[17] in the therapeutic trials, namely, 0.25 Gm. (commercial product, 73% NaF) per pound of body weight, removed 98 per cent (84) of 85 nodular worms from 5 pigs. While this dosage appears to be well tolerated, it is higher than that required for efficient removal of ascarids.

It should be noted, moreover, that information on the efficacy of sodium fluoride is not only adequate for significant comparisons with oil of chenopodium and phenothiazine, but is also more extensive than that on either of the older drugs. The literature, as reviewed in a recent publication,[5] discloses that oil of chenopodium in critical trials removed 182 (74%)

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*An additional reference[18] is significant in this connection but contains no specific data. In table 1, C, of the present paper, data ascribed to this source are from unpublished records.

†Roberts[17] treated groups of 2 to 9 pigs but did not state the number of groups containing 2 animals.

of 245 ascarids from 59 pigs, and that phenothiazine removed 736 (36%) of 2,022 ascarids from 83 test pigs. A significant action of sodium fluoride, in addition to its afore-mentioned efficacy of 95 per cent, is its removal of immature, as well as mature, roundworms.

Data on the efficacy of sodium fluoride when administered by methods other than in feed mixture are limited, mainly because such other methods of giving the chemical appear to cause unusual risk to the host.

In passing, note should be taken of the ascaricidal efficacy (93%) of sodium fluoride when administered at the currently accepted rate of 1 per cent in feed for one day. This may not prove to be the optimum rate of dosage, yet a breakdown of the experience of those who have reported critical trials indicates that all have found the regimen effective, but suggests by its variability (averages 87 to 97%) that reduction in the absolute dose below that which ordinarily operates under this regimen may result in less efficient removal (table 2). It may be significant, however, that all the ascarids were removed from 156 (86%) of the 182 test animals treated at the 1 per cent level. The other 26 pigs (14%) harbored 68 worms at autopsy. The lowest determined individual efficacy in this group was 50 per cent.

TABLE 1—Anthelmintic Efficacy of Sodium Fluoride in Swine
Administration in Feed to 319 Pigs

NaF Dose (%)	No. Pigs	Worms Removed	Worms Left	Efficacy (%)	Ref. No.
A. Large roundworms.					
2.0—1 day	8	137	0	100	10
1.5—1 day	58	203	1	99	10
1.0—3 days	5	65	2	97	10
1.0—2 days	10	62	2	97	1
1.0—1 day	182	926	68	93	1,2,3,10,15
0.3—3 days	10	292	4	98	11
0.1-0.25 Gm./lb.	35	579	28	95	17
	308	2264	105	95	
B. Stomach worms.					
2.0	6	2492*	21	99	10
1.5	9	720*	24	96	10
1.0	6	251*	153	62	10
0.1-0.25 Gm./lb.	22	168**	74	69	17
	43	3631	272	93	
C. Nodular worms.					
2.0	7	552	924	37	10
1.5	10	1571	2141	42	10
1.0	7	69	1383	5	10
0.1-0.25 Gm./lb.	29†	230	1150	16	17,18
	53	2422	5598	30	
D. Whipworms.					
Various rates	37	302	1191	20	10,17
E. Thorn-headed worms.					
Various rates	6	0	25	0	15,17

Ascarops strongylina
**A. strongylina* and *Physocephalus sexalatus*
†Includes 3 pigs at 0.75% in feed.[18]

Toxicity.—Significantly, none of the aforementioned authors, except ourselves,[10] experienced death losses in either the therapeutic or toxicity trials with sodium fluoride. The details of our losses in both categories have been fully described (*loc. cit.*). Briefly summarized, there were 2 fatalities, on the fourth and sixth days, respectively, among 52 pigs given 1 per cent in feed for one day. The pathologic findings in the 2 cases were different, and pneumonia was the evident cause of 1 of the deaths. Moreover, the pigs were a constitutionally inferior lot that showed high mortality during the interval when they were held before experimental use. There were 2 other deaths from pneumonia during the other therapeutic trials—1 on the fourth day after treatment with 1 per cent in feed for three days, and 1 on the fifth day after treatment at the 1.5 per cent level for one day. In specific toxicity trials, 1 of 2 pigs died on the sixth day after being fed a ration containing 4 per cent of the chemical, but pneumonia was again the only discernible cause of death. Two pigs fed a 5 per cent concentration died in one to three days. There were marked hemorrhagic gastroenteritis and generalized congestion. It is evident, therefore, that a ration containing 5 per cent of the chemical is extremely toxic. With 1 possible exception (see p. 137 of ref. 10), however, there have been no deaths from fluoride poisoning when the chemical was given in feed at a level of 2 per cent or less or at a rate of 0.25 Gm. or less per pound of body weight, and the number of such treatments exceeds the number of animals employed in the therapeutic trials. For example, Roberts[17] used only 43 pigs in specific therapeutic trials, as heretofore described, yet he gave 318 treatments to a total of 282 pigs. In all the critical studies to which reference has been made, 632 treatments with sodium fluoride were given to 572 pigs. Excluding the 3 deaths from specific toxicity trials at levels of 4 and 5 per cent, there were 4 deaths from all causes following the administration of 629 treatments to 569 pigs. Inasmuch as at least 3 of the 4 deaths appeared not to be due to sodium fluoride, mortality from the medication at all therapeutic levels approximated a rate of 1.6 per 1,000. At optimum dosages, therefore, the mortality should be lower.

As expected, instances of mild intoxication, manifested either clinically or pathologically, or both, were numerous compared with death losses. Vomition, soft feces, and sometimes diarrhea were temporary symptoms in from 6 to 10 per cent of the treated animals, the exact percentage being impossible to deter-

mine because of the large numbers of pigs used in group treatments. The vomition seemed unaccompanied by nausea, since there was little, if any, evidence of discomfort or of disinclination to resume eating. Apparent inappetence, manifested by reduced food intake and occasional refusal to eat, was common. The reaction is due to the unpalatability of the medicated feed and is desirable to the extent that it affords some natural protection against overdosage. True inappetence occurred more rarely, and probably only in animals suffering inflammatory change in the alimentary tract.

TABLE 2—Ascaricidal Efficacy at 1 Per Cent for One Day

Data of different investigators				
		Worms		
Investigators	No. pigs	Removed	Left	Efficacy
Allen[1]	49	235	35	87
Allen and Andrews[2]	2	44	6	88
Allen and Jones[3]	59	260	8	97
Habermann, Enzie, and Foster[10]	52	262	8	97
Morgan and Grummer[15]	20	125	11	92
	182	926	68	93

Such inflammation, occurring as gastroenteritis, colitis, or congestion, was found in about 3 per cent of the animals when autopsied one to seven days after treatment. Three or 4 animals were killed *in extremis*. The condition of at least 2 of these was ascribed to general debilitation (*see* p. 136 of ref. 10), and in only 1 case were the symptoms suggestive of fluoride poisoning (*see* p. 88 of ref. 17).

Special Considerations Regarding Toxicity.—The experiences described show clearly that the use of the sodium fluoride treatment involves some risk to the animals. It is, nevertheless, as shown elsewhere,[6] a safer medication than either oil of chenopodium or phenothiazine and is of sufficient safety to warrant general use.

Several special problems, however, are necessarily involved in a full appraisal of the safety of the treatment under discussion. It is evident, for example, that any inflammatory condition of the alimentary tract is a contraindication to the administration of sodium fluoride. It may be best, also, to withhold the medication from pregnant sows, although several have been treated with no apparent injury.[12] Because of the effect of fluorine upon blood calcium, low calcium diets or hypocalcemia may predispose to intoxication by the medication, although no relationship of this nature has become evident. This factor is probably of little or no consequence under ordinary circumstances, as in the case of market hogs; yet, under conditions such as pregnancy and lactation, fortification against calcium depletion might prove beneficial. In passing, acknowledgment should be made of the opposing view that calcium restriction enhances the efficacy of the medication.[7] Experience does not seem to support this view, and safety is uppermost among the criteria of usefulness of any anthelmintic.

The human element is also involved. Inasmuch as most hogs are destined for human consumption, treatment of these animals with sodium fluoride would be unsafe if it increased the fluorine content of the tissues above limits occurring naturally. When our tests were initiated, analyses were obligingly made by chemists of the Meat Inspection Division of the USDA. Their conclusion was that the minute amounts of fluorine found in the samples could have no significance from a consumer standpoint. A similar conclusion, on the basis of specific analyses, was reached by Morgan and Grummer.[15] Finally, one must consider the possibility that a wider use of sodium fluoride as a pest control chemical would inevitably increase the risk of accidental poisoning of animals and man. The only effective way to guard against this is to emphasize repeatedly the poisonous nature of the chemical, of which, fortunately, most farmers are aware. Paradoxically, such caution is most necessary when reference is made to the safety of the medication.

Administration.—Ease of administration, next to safety and efficacy, is a criterion of the usefulness of any anthelmintic. In the case of medication for the removal of ascarids from pigs, the method of choice should obviate individual handling of animals, as well as preparatory fasting and purgation. Administration in feed achieves these objectives, and little attention has been given to other methods of dosing. Some data are available, however, which should be made a part of the record because of numerous questions that have arisen. Moreover, treatments are largely established by use, and diverse methods of administering sodium fluoride to hogs have come into the picture since the original description of the treatment. It has become important, therefore, to consider critically the underlying reasons and data that contribute to an understanding of the safest, simplest method.

Capsules.—The literature contains no account of experience with this method of administration, but some trials that we have made may be summarized (table 3, A). The chemical was given in capsules to 8 pigs, in dosages of 0.1 and 0.15 Gm. per pound

of body weight, after the pigs had eaten a portion of their regular feed. The smaller dosage removed 70 of 79 ascarids (88%) from 4 pigs, but vomition and marked salivation occurred in 2 cases. In doses of 0.15 Gm. per pound, the chemical removed all of 17 ascarids from 3 pigs; an additional pig harbored no ascarids. However, 2 pigs vomited and 1 died (pig 297, *see* table 3). These trials showed an average efficacy of 90 per cent against ascarids but no efficacy against the other species. The frequency and severity of reactions, at dose rates that are well tolerated in feed, as well as the obvious necessity of handling the animals individually make it appear that administration of the chemical in capsules has almost certainly no special advantage, and probably involves undue risk of intoxication.

Stomach Tube.—Roberts[17] dosed 4 pigs

TABLE 3—Sodium Fluoride Administered in Capsules and by Stomach Tube

Method	Pig. (No.)	Wt. (lb.)	Dose (Gm.)	Gm./lb.	Parasites	Removed	Left	Efficacy (%)
A. Capsule	286	29.0	2.9	0.1	A.	0	3	0
	287	26.0	3.9	0.15	A.	?	0	?
					S.	2	107	1
	294	38.0	3.8	0.1	A.	25	1	96
					W.	0	17	0
	295	34.0	5.1	0.15	A.	9	0	100
					W.	0	8	0
	296	45.0	6.75	0.15	A.	1	0	100
					N.	0	14	0
					W.	0	3	0
	297	32.0	4.8	0.15	A.	7	0	100
					N.	0	119	0
					W.	0	8	0
	299	35.0	3.5	0.1	A.	22	0	100
					W.	0	3	0
	300	35.0	3.5	0.1	A.	23	5	82
					N.	0	73	0
					W.	0	31	0
B. Stomach tube	288	32.0	6.4	0.2	--	–	–	–
	289	43.0	6.45	0.15	--	–	–	–
	290	20.0	4.0	0.2	--	–	–	–
	291	20.0	4.0	0.2	A.	6	5	54
					W.	0	2	0
	292	48.0	9.6	0.2	A.	15	2	88
					W.	0	12	0
	293	33.5	6.7	0.2	A.	7	6	53
					W.	0	29	0
	298	18.0	1.8	0.1	A.	61	6	91
					S.	0	3	0
					N.	0	31	0
					W.	0	19	0

A. = Ascarids, S. = Stomach worms, W. = Whipworms, N. = Nodular worms.

Remarks:

Pig 286: Vomited about thirty min. after treatment.
Pig 287: Vomited on third day; ascarid ova on fecal examination before treatment.
Pig 296: Partially healed hemorrhagic area about 3 in. in diameter on stomach mucosa.
Pig 297: Vomited; died on third day; hemorrhagic area about 2 in. in diameter on stomach mucosa; marked congestion of liver & kidneys; small int. hemorrhagic; intussusception in two areas; poor risk for medication—clinical bronchitis before treatment.
Pig 299: Mucus in feces on second and third days.
Pig 300: Vomited about one hour after treatment.
Pig 288: Vomited several times about thirty min. after treatment.
Pig 289: Found dead on second day; probably froze to death.
Pig 290: Vomited; died about two hrs. after treatment; marked hemorrhagic gastroenteritis; marked congestion of liver and kidneys. Gastroenteritis may have been present at time of treatment.
Pig 291: Vomited; off feed for two days; autopsied three days after treatment, organs apparently normal. Low efficacy may have been due to short post-treatment period; the 6 ascarids were found in the large intestine.
Pig 292: Vomited.
Pig 293: Vomited.
Pig 298: Vomited.

by this method. Two were given 0.3 Gm. per pound of body weight, and 2 others 0.25 Gm., the chemical being given after a meal and washed down the tube with water. The treatments were followed in all cases by violent vomiting for five to six hours, although the pigs returned to apparent normalcy in one to four days. No data were obtained on the efficacy of the treatments. In trials by us (table 3, B), the chemical was given in aqueous solution to 7 pigs in dosages from 0.1 to 0.2 Gm. per pound of body weight. Treatment was given after part of the regular feed had been consumed, and the pigs were returned to regular feed immediately. From 3 of the 4 pigs on which it was possible to carry out critical tests, the treatment removed 83 of 97 ascarids (85%; 1 pig had none), but showed no action against other species. Six pigs vomited and 2 died. Circumstances indicated that 1 death may have been accidental, and that the chemical may have been only a contributing cause of death in the other case (pigs 289 and 290, *see* table 3). Although this experience involves only 11 pigs, the results seem to justify the conclusion that administration by stomach tube is dangerous as well as objectionable on practical grounds.

Feed.—Emphasis has been placed on the administration of the chemical in dry feed, and the safety, efficacy, and simplicity, as well as the underlying reasons of this regimen require little additional comment. A wide field of experience supports this regimen,[19] and, experimentally, many kinds of ground feed mixtures have been employed as vehicles for the chemical with results, in all cases, about equally satisfactory. A method devised by Roberts,[17] and presumably used with success in Australia, consists in giving the chemical at rates from 0.1 to 0.15 Gm. per pound of body weight, the total dose for a group of pigs being mixed with that amount of feed, which allows about 1 lb. of feed for each 25 lb. of hog. Experimentally, the mixture was fed moist, and the results were satisfactory. Under this regimen, the concentration of the chemical in feed is somewhat lower than 1 per cent, but the amount of medicated feed consumed by hogs is somewhat larger than that consumed when the chemical is fed at the 1 per cent level.

Circumstances required that consideration be given to the practicability of using other hog feeds, including milk, as well as those designated as slop, swill, and garbage. Of these, milk is definable and meets the requisites of satisfactory constancy in weight and uniformity of consumption, but the others are indefinable, of diverse composition, and the amounts that hogs consume are highly variable. In spite of the evident contraindications to formulating such feeds, some tests were made to determine if they were unsuitable for reasons of toxicity, as had been suggested by field experience.[19]

In trials with milk, 4 pigs were tested on a formula comparable to that used with dry feed, namely, 1 per cent of sodium fluoride by weight. It was recognized, of course, that the consumption of milk by weight is much greater than that of dry feed, but the fact was borne in mind that sodium fluoride renders feeds relatively unpalatable to hogs. Moreover, in field use, there appeared to be the tendency to employ one and the same formula, regardless of the type of feedstuff. Consumption of the medicated milk was only about 15 per cent of normal, but the estimated dosage was about 0.33 Gm. per pound of body weight. All the pigs vomited and suffered some discomfort, and all showed some injection of the gastric or intestinal mucosa when autopsied one to four days after treatment. No ascarids were present in 3 of the 4 pigs, but, significantly, the fourth pig, which was autopsied the day after treatment, harbored 16 ascarids that were apparently unaffected by the medication.

Three pigs were given the chemical in a slop prepared with 1 part by weight of ground feed and 2 parts of skimmed milk. To 1 pig, weighing 125 lb., medication was given at the 1 per cent level. About 32 Gm., or 0.25 Gm. per pound, of sodium fluoride were consumed over a 24-hour period. No ascarids were present in this animal, but the reaction to the medicament, both clinically and pathologically, was severe. On

the second and third days, there were marked inappetance, weakness, and posterior paralysis. On the fourth day, however, the animal was again in good condition, but at autopsy on the fifth day, there were congestion of the kidneys and gastric mucosa and an excessive amount of mucus in the small intestine. For the other 2 pigs, treated together, the concentration of drug in the slop was reduced to 0.33 per cent. There were no inappetence and no vomition, and about 12.5 Gm. of the chemical were consumed in a 24-hour period. This was a dosage of somewhat less than 0.15 Gm. per pound of body weight, since the pigs weighed 30 and 55 lb. Two of 3 ascarids were removed by the medication but, at autopsy on the fifth day, 1 pig revealed mild congestion of the stomach, small intestine, and kidneys.

The experiences described support the view that pigs are quite tolerant of sodium fluoride but dangers are sufficient to warrant the tentative conclusion that administration of the chemical in any vehicle other than dry, ground feed, unless in accordance with the slightly modified method described by Roberts,[17] is likely to be unsatisfactory from standpoints of both safety and efficacy. On the one hand, there appears to be nothing to recommend these other methods, while on the other hand, investigational and field experience[19] to date, however inconclusive, appear to support the method of administration in current use.

Concentration of Fluoride in Feed.—Experience amply confirms the safety and efficacy of administering the chemical in feed at a level of 1 per cent, but there is little, if any, evidence that this is the optimum rate. Moreover, any refinement must be toward reduction of the fluoride concentration, since an increase would achieve little in efficacy and sacrifice much in safety. At present, no data are available which justify reduction, but concentrations of 0.75 per cent have been used successfully in a few instances.[18] In addition to other considerations, it should be borne in mind that the absolute dose of the chemical is determined not only by the concentration at which it is fed but also by the amount of feed consumed. When the 1 per cent level is employed, feed consumption is from one-fourth to one-half of normal, and considerable significance has been attached to the unpalatability factor. The feeds containing 1 per cent, for example, appear to be sufficiently unpalatable to cause self-imposed restriction of intake, with consequent protection against overdosage. On the other hand, such feeds contain a sufficient concentration of the chemical to cause vomition when there is occasional overindulgence, and this also affords protection against overdosage. In any refinement of dose rate, these natural safeguards against intoxication must be preserved or compensated by advantages of comparable magnitude. The question of optimum dose rate is consequently difficult and complicated but is important and should receive further study.

SUMMARY

In therapeutic trials with sodium fluoride administered in ground feed, the combined data from seven groups of investigators show that the chemical removed 2,264 of 2,369 ascarids (95%) from 308 pigs and 3,361 of 3,903 stomach worms (93%) from 43 pigs. The chemical was only slightly effective or entirely ineffective against nodular worms, whipworms, and thorn-headed worms. At the provisional dose rate of 1 per cent in dry, ground feed, the efficacy against ascarids was 93 per cent and against stomach worms, 62 per cent. Untoward reactions were infrequent; vomition and soft feces, both of temporary duration, occurred in about 10 per cent of the cases. Concentrations of 4 and 5 per cent of sodium fluoride in feed were extremely toxic, although, at lower concentrations, doses up to 1 Gm. per pound of body weight, about 10 times the therapeutic level, did not cause serious reactions. In experimental and field trials, administration of the chemical in any other vehicle than ground feed appeared to lack special advantages and to impose undue risks of intoxication. More experimental data are needed to determine the optimum rate of administration, but the experience described supports fully the view that the administration of sodium

fluoride in dry, ground feed at a level of 1 per cent for one day is a safe, effective, simple, and economical regimen of treatment.

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The Serologic Differentiation of Salmonella Pullorum Forms

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DIFFERENTIATION of "standard" and "variant" strains of *Salmonella pullorum* is based on the form variation of Kauffmann. In *S. pullorum*, this variation affects antigen XII which is divisible into three portions—XII_1, XII_2, and XII_3. Antigens XII_2 and XII_3 are subject to variation in many Salmonella types of groups B and D of the Kauffmann-White classification. As a rule, this variation is expressed in the relative amounts of those two antigens in a given culture at a given time. Curiously, a culture which contains a large amount of XII_2 usually contains little XII_3 while the presence of a large amount of XII_3 usually means that XII_2 is present in a minimal amount.

When *S. pullorum* cultures lose this power of form variation and become stabilized, standard and variant strains are produced. The majority of *S. pullorum* cultures contain a large proportion of XII_3 and a small proportion of XII_2, thus these are called standard strains. In variant strains, the proportions of these antigens are reversed.

MATERIALS

In order to differentiate between the standard and variant forms of *S. pullorum*, it is essential to have two different serums on hand —one containing the XII_2 agglutinin and another containing the XII_3 agglutinin. Neither serum should contain the IX agglutinin since the IX antigenic factor is common to both forms of *S. pullorum*.

PREPARATION OF IMMUNE SERUM

A XII_2 serum can be prepared by inoculating a rabbit or a hen (the agglutinin response of the rabbit is greater in this case) with a heat-treated broth culture of *Salmonella reading* which has a well developed XII_2 antigen. Generally, four intravenous inoculations are given, starting with 0.5 cc. and gradually increasing to 1.5 or 2.0 cc. The inoculations should be made at intervals of three to four days. A satisfactory inoculum may also be prepared by washing the 24-hour growth from a nutrient agar slant with physiologic saline. The turbidity is adjusted to 1.0 on McFarland's nephelometer scale and then boiled for two hours to destroy the H antigens.

When a satisfactory titer is obtained, the blood is collected (5 to 6 days after the last inoculation) and allowed to clot normally. The serum is allowed to exude (this process can be hastened by centrifugation) after which it is poured off and allowed to stand at about 4 C. for twelve hours, or overnight, and then cleared in the centrifuge. With 0.2 per cent tricresol added as a preservative, an immune serum will retain its potency for a long time if it is stored at about 4 C.

PREPARATAON OF A XII_2 SERUM

S. reading immune serum contains agglutinins IV, XII_1 and XII_2. The XII_1 agglutinin must be removed since both forms of *S. pullorum* possess this antigen. In order to remove the XII_1 agglutinin, the serum is absorbed with *Salmonella paratyphi* A var. *durazzo* which possesses antigens II, XII_1, and XII_3.

The absorbing suspension is prepared by seeding *S. paratyphi* A var. *durazzo* onto Petri dishes (100 mm. in diameter) containing 40 to 50 cc. of meat infusion agar, or nutrient agar containing 2 per cent bacto peptone and 2 per cent agar. The seeded Petri dishes are incubated twenty-four hours at 37 C. after which the growth is washed off with 0.5 cc. of normal saline per dish and added to the serum. Usually the growth from five to six Petri dishes is required to remove the XII_1 agglutinin from 1 cc. of *S. reading* immune serum. The serum and bacteria are mixed thoroughly and incubated in a water bath for two hours at 50 C. and then centrifuged until clear. The clear serum is decanted.

The continued interest and support of Professor E. H. Garrard, head, Department of Bacteriology, Ontario Agricultural College, in all phases of this work has been very gratifying.

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When the absorption is complete, the serum should contain agglutinins IV and XII_2. The IV agglutinin will not cross react with *S. pullorum* hence its presence is disregarded. This serum may be further diluted with physiologic saline until a clear reaction with the homologous antigen takes place in thirty seconds to one minute. The absorbed serum should be preserved by adding a slight excess of chloroform and mixing it carefully. It is then corked and stored in the refrigerator at 4 C. Such a serum will retain its potency for only a few months and, hence, it is useless to absorb serum in quantities that are not going to be used within this time.

An alternative method for the preparation of a XII_2 serum is by the inoculation of a horse or rabbit with Gwatkin's Proteus I. This serum will react strongly with variant strains of *S. pullorum* while there is generally little reaction with the standard strains.

PREPARATION OF A XII_3 SERUM

The procedure for the preparation of a XII_3 serum is essentially the same but performed in the converse, *i.e.*, a *S. paratyphi* A var. *durazzo* immune serum is absorbed with *S. reading*. Such a serum will contain II, XII_1, and XII_3 agglutinins. The XII_1 agglutinin is removed by *S. reading* while the II agglutinin is disregarded in this serum since it will not agglutinate *S. pullorum*.

Rabbits or birds for immune serum production should be healthy and negative to *S. pullorum*. The serologic response of birds 5 to 10 months of age is preferable to that of older ones.

DIFFERENTIAL REACTIONS AND DISCUSSION

The XII_2 and XII_3 serums should not be used until the identity of the culture being studied is assured, *i.e.*, the culture must be identified as *S. pullorum* before its antigenic characteristics are investigated.

The absorbed serums, diluted 1:5, may be used conveniently in slide agglutination tests. A small amount of growth rubbed up in a drop of saline should be divided into two parts. A loopful of XII_2 serum should be added to one portion, a loopful of XII_3 serum to the other. The slide is then tilted until agglutination is observed.

Variant cultures usually agglutinate well in XII_2 serum, but slowly and slightly with XII_3 serum. With standard strains, these reactions are reversed. Since standard strains are not entirely devoid of XII_2 and variants not entirely devoid of XII_3, some degree of cross reaction must be expected.

It sometimes happens that cultures are agglutinated well both by XII_2 and XII_3 serums. These are the so-called intermediate strains. When such cultures are plated and examined, they often yield some colonies which agglutinate well in XII_2 serum and others which agglutinate in XII_3 serum. This is the typical picture of form variation as it is known in the Salmonella group. Other cultures yield only colonies each of which is agglutinated by both serums. Such a situation is interpreted as form variation so active that both XII_2 and XII_3 forms are produced in the individual colonies. If such cultures are transferred several times, they will usually cease to vary so actively and behave as typical XII_2 or XII_3 cultures.

Since the differentiation of standard and variant strains is based on a variational phenomenon, a diagnosis must be made with a certain degree of caution and discretion. Form variation being what it is, a diagnosis of a variant strain should not be based on the examination of a single colony or even several colonies. From such a cursory examination, one is justified in saying only that a culture is predominantly in the XII_2 or XII_3 form. In order to state positively that a stabilized variant strain of *S. pullorum* is involved, a large number of colonies (at least 100) must be examined to ascertain that the culture actually has become stabilized in the XII_2 form. If these precautions are kept in mind, the disagreements between the results of different laboratories will be minimized.

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Immunity Studies in Avian Cecal Coccidiosis

I. The Value of Drugs to Establish Immunity in Young Chickens

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IMMUNITY to avian cecal coccidiosis may be acquired in chicks either from gradual ingestion of sporulated oöcysts or following recovery from a severe attack of the disease. Under natural conditions, following a severe attack of the disease there is usually a solid immunity but, when there is a gradual ingestion of sporulated oöcysts, the degree of immunity or resistance varies. In recent years, various drugs, especially the sulfonamides, have been used to prevent coccidiosis and at the same time allow chicks to build up enough immunity to protect them against a later attack by the disease.

Tyzzer[1] reported that severe infections with *Eimeria tenella* gave well marked and prompt immunity, but slight infections with a few oöcysts were not sufficient to protect against massive doses of the same species. Beach and Corl[2] also reported that chicks that survive an acute outbreak of coccidiosis are resistant to subsequent infection. Farr[3] found that a severe case of coccidiosis resulting from a single, heavy inoculation did not produce effective resistance to reinfection. Swales[4] stated that no birds infected and treated in their laboratories developed acute coccidiosis a second time even though they were continually exposed to the infection up to the time of marketing.

Jankiewicz and Scofield[5] established an effective resistance and, in some cases, a total immunity to further infection with *E. tenella* by the capsule feeding of heated oöcysts. Johnson[6] reported that "results lend encouragement to feeding sporulated oöcysts in wet mash as practical means of immunization." Waxler[7] found that chicks infected with standardized, x-ray-attenuated doses of *E. tenella* resisted a subsequent heavy infection almost as well as the survivors of a severe infection produced by untreated cultures. Waxler and Herrick[8] infected chickens with x-ray-attenuated oöcysts, measured the resistance established by the hemoglobin level after infection, and reported the resistance almost as great as that of survivors that had been originally infected with untreated oöcysts.

Practically all the sulfa drugs[9] have been used experimentally in the treatment and prevention of avian cecal coccidiosis. Sulfaguanidine has been tested extensively by many workers. Seeger[10] found that sulfaguanidine gave high protection against losses in chicks receiving heavy doses of sporulated *E. tenella* oöcysts and that survivors of the initial inoculation with and without treatment were found to be completely immune to a second inoculation.

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Sulfaquinoxaline furnished by Merck & Co., Rahway, N. J.

Farr and Allen[11] reported that chicks protected from an initial infection of cecal coccidiosis by sulfaguanidine were highly susceptible to reinfection one month after cessation of treatment. Later, the same authors[12] reported that chicks fed smaller concentrations of the drug and held on infective litter for twenty-one days after cessation of treatment were fully as resistant to subsequent infection as the surviving untreated controls.

Sulfamethazine and sulfamerazine have also been used extensively in the treatment and prevention of avian cecal coccidiosis. Horton-Smith and Taylor[13] reported that sulfamethazine in the feed gave almost complete protection against avian cecal coccidiosis; and, in a later paper,[14] that sulfamethazine in the drinking water also gave excellent results, and the chicks possessed a strong immunity. Swales[15] suggested new methods of controlling and creating an immunity to later attacks of cecal coccidiosis in chicks by the use of sulfamethazine and sulfamerazine and their soluble salt preparations in the feed or the drinking water.

Hardcastle and Foster[16] used borax as a protective drug against cecal coccidiosis and found that borax in the drinking water considerably enhanced its efficacy.

Delaplane, Batchelder, and Higgins[17] reported on the use of sulfaquinoxaline in the prevention of coccidiosis and showed that the drug does not interfere with the development of a desirable degree of resistance to coccidiosis. Grumble, Delaplane, and Higgins[18] also found that sulfaquinoxaline can be used effectively to prevent mortality during the time required for immunity to develop.

MATERIAL AND METHODS

New Hampshire and White Leghorn day-old chicks were reared coccidia-free in wire-bottomed batteries, until they were ready for use in the experiments. In each of the experiments, the chicks were from the same hatch and divided evenly into groups on the basis of live weights and sex. All chicks that died or were killed were examined for lesions of coccidiosis.

Fecal examinations, using the sodium nitrate flotation method, were conducted on all groups of chicks used in the experiments to further assure they were free of coccidia. The chicks were experimentally infected by using a pipette to deposit the sporulated *E. tenella* oöcysts in the crop. The naturally infected chicks were placed in outside pens where they ingested the oöcysts from contaminated ground.

The outside yard pens used in the natural infection experiments were 14 ft. square, completely enclosed, sides and top, with 1 in. square mesh wire netting, and the same type wire was buried 2 ft. to make the pens rat proof. The pens were so constructed that wire

TABLE I—Results from Treatment of Chickens with Certain Compounds for Seven Consecutive Days Following Inoculation with Eimeria Tenella

Group	Treatment	No. of chicks	Age (weeks)	No. of oöcysts	Died acute coccidiosis	Ave. wt. gains 35 days (lb.)
1	0.2% Sodium sulfamerazine in drinking water	20	4	150,000	0	1.05
2	0.2% Sodium sulfamethazine in drinking water	20	4	150,000	1	1.18
3	0.1% Sulfaquinoxaline in feed	20	4	150,000	0	1.40
4	0.2% Sodium borate in drinking water	20	4	150,000	3	1.13
5	Susceptibility controls	20	4	150,000	15	1.01
6	Normal controls	25	4	None	0	1.47

Note: The surviving chicks from groups 1, 2, 3, 4, and 5, were challenged with 150,000 sporulated *E. tenella* oöcysts four weeks after the original inoculation. On the fourth day, there were traces of blood in the droppings from groups 1 and 2 but no evidence of cecal hemorrhage on postmortem examination one week after the challenging dose. Groups 3, 4, and 5 showed neither clinical nor postmortem evidence of coccidiosis.

partitions could be placed inside in order to divide the pen into four small compartments 7 by 7 ft. Small, movable wire cages with open bottoms were used in the larger pens to hold the control chicks. These small cages, containing the control chicks, were moved around the larger pens systematically at half-hour intervals during the day so that the ground in the entire larger pen was covered by the control chicks.

To assure naturally contaminated outdoor pens, different groups of chicks 4 to 8 weeks of age, were infected and placed in the pen for a period of six weeks so as to spread oöcysts on the ground. A total of 98 birds was used in each pen for infectivity production. Periodic fecal checks from cecal evacuations and from the ground showed the chicks were spreading *E. tenella* oöcysts consistently during the six weeks.

The drug solutions were prepared fresh and placed in porcelain or glass containers.

EXPERIMENTAL PROCEDURE

To study the extent of immunity or resistance developed to further attacks of cecal coccidiosis after a clinical attack of the disease, each of 120 4-week-old New Hampshire chicks, was inoculated with 150,000 sporulated *E. tenella* oöcysts. Forty-two chicks died from acute coccidiosis, and the remainder of the chicks showed clinical evidence of the disease. The chicks were first kept on fresh, clean shavings and later, as the groups became smaller, were placed in small houses with clean straw litter. Two weeks following the original infection, groups of surviving chicks were tested for immunity or resistance. At the beginning of each week, a group of the survivors, along with controls, were challenged by depositing 150,000 freshly sporulated *E. tenella* oöcysts in the crop; during the week they were held in small wire-bottomed cages and watched for any evidence of recurring coccidiosis; at the end of the week an autopsy was conducted. These tests were continued for ten weeks or until the last group of chicks had reached the age of 14 weeks. None of the surviving groups, from the original inoculation that were challenged, showed any clinical or postmortem evidence of recurrence of coccidiosis. The controls used along with the tests had an 80 per cent mortality.

The drugs used in the following experiments were those that had previously been tested and found to have some value in the treatment or prevention of cecal coccidiosis.

Experiment 1.—Five groups of 20 New Hampshire 4-week-old chicks were inoculated with 150,000 freshly sporulated *E. tenella* oöcysts. Immediately following the inoculations, four of the groups were put on a test drug for seven days. The drugs used were 0.2 per cent sodium sulfamerazine, 0.2 per cent sodium sulfamethazine, 0.3 per cent sodium borate in the drinking water, and 0.1 per cent sulfaquinoxaline in the feed. One untreated group was used as susceptibility controls. Another group of 26 chicks, not inoculated, was used as normal controls to determine weight gains. Challenging doses of 150,000 sporulated *E. tenella* oöcysts were given four weeks after the original inoculation.

The chicks given sodium sulfamerazine, sodium sulphamethazine and sodium borate (table 1) showed clinical symptoms of cecal coccidiosis by copious hemorrhagic cecal evacuations. Death losses from acute coccidosis occurred in the groups given sodium sulfamethazine, sodium borate, and in the controls. The chicks in the group fed sulfaquinoxaline were not clinically sick and made the best weight gains. In the sodium sulfamethazine group, 1 chick died on the morning of the twenty-eighth day after the

TABLE 2—Results from Treatment of Chickens with Certain Compounds for Three Consecutive Days following First Clinical Appearance of Coccidiosis after Inoculation with Eimeria Tenella

Group	Treatment	No. of chicks	Age (weeks)	No. of oöcysts	Died acute coccidiosis	Ave. wt. gains 35 days (lb.)
1	0.2% Sodium sulfamerazine in drinking water	20	4	150,000	6	1.29
2	0.1% Sulfaquinoxaline in feed	20	4	150,000	3	1.13
3	0.3% Sodium borate in drinking water	20	4	150,000	14	...
4	Susceptibility controls	20	4	150,000	15	1.00
5	Normal controls	27	4	None	0	1.40

Note: The surviving chicks from groups 1, 2, and 4, challenged with 150,000 sporulated *E. tenella* oöcysts four weeks after the original inoculation, showed neither clinical nor postmortem evidence of recurrence of coccidiosis.

original inoculation. The cecums were ruptured, gangrenous, and contained very large necrotic cores.

Four weeks following the original inoculation, all the surviving chicks from groups 1, 2, 3, 4, and 5, along with coccidia-free controls, were given a challenging dose of 150,000 freshly sporulated *E. tenella* oöysts. The controls showed an 80 per cent mortality from this inoculation. On the fourth day following inoculation, traces of blood were seen in the droppings from chicks that had been given sodium sulfamerazine and sodium sulfamethazine. The remaining groups showed no evidence of recurrence of clinical coccidiosis. On postmortem examination one week following the challenging dose, there was no evidence of cecal hemorrhage in any of the groups. One chick in each of the groups 1, 2, and 3 showed very large, old, necrotic, cecal cores, and several chicks in each group showed medium and small cores. The chicks with large and medium-sized cores made poor weight gains.

Experiment 2.—Four groups of 20 New Hampshire chicks 4 weeks old were inoculated with 150,000 sporulated *E. tenella* oöcysts. After the first symptoms of cecal coccidiosis, three groups were given the test drugs for three consecutive days, and the other group was used as susceptibility controls. The drugs used were 0.2 per cent sodium sulfamerazine, 0.3 sodium borate in the drinking water, and 0.1 per cent sulfaquinoxaline in the feed. Another group of 27, not inoculated, was used as normal controls. Challenging doses of *E. tenella* oöcysts were given four weeks after the original inoculation.

The first symptoms of cecal coccidiosis, namely depression and hemorrhagic cecal evacuations, occurred in the ninety-fourth hour after the inoculation. All the groups were then given the test drugs. When the sulfaquinoxaline in the feed was given to group 2, it was noticed that 2 of the chicks refused to eat; these were found dead from acute coccidiosis the following morning. Sodium borate in the drinking water showed the poorest results. After 14 deaths in this group, the test was discontinued. One chick died in the sulfaquinoxaline group on the twenty-seventh day with a ruptured gangrenous cecum containing a very large core. The surviving chicks from groups 1, 2, and 4 were challenged with 150,000 freshly sporulated *E. tenella* oöcysts four weeks after the original inoculation. One week later, the chicks were killed and autopsied, and no evidence of cecal hemorrhage was observed. Susceptibility control chicks used along with the challenged chicks showed 80 per cent mortality. In group 1, 1 chick had a large, old, necrotic cecal core and several had small cores; in Group 2, 2 chicks had very large, old, necrotic cecal cores and several had small cores.

Experiment 3.—In this experiment, five groups of 12 White Leghorn chicks 6 weeks old were put in naturally contamined pens. As soon as the chicks were placed in the infected pens, the test drugs were given. The drugs used were 0.3 per cent sodium sulfamethazine, 0.3 per cent sodium sulfamerazine, 0.3 per cent sodium borate in the drinking water, and 1.0 per cent sulfaguanidine in the feed. The controls were kept in small movable pens and moved around the larger pens to a new area at half-hour intervals so that the entire ground was covered. At the same time the

TABLE 3—Results from Treatment of Chickens with Certain Compounds for Seven Consecutive Days Following Natural and Experimental Infections

Groups	Treatment	Pen	No. of chicks	Age weeks	Died, acute coccidiosis during treatment	Died, acute coccidiosis within four days after treatment discontinued
1	0.3% Sodium sulfamethazine in drinking water	A	12	6	0	2
		B	3	6	0	0
2	0.3% Sodium sulfamerazine in drinking water	A	12	6	0	0
		B	3	6	0	0
3	0.3% Sodium borate in drinking water	A	12	6	0	0
		B	3	6	0	0
4	0.1% Sulfaguanidine in feed	A	12	6	0	3
		B	3	6	0	0
5	Susceptibility controls	A	12	6	6	0
		B	3	6	3	0

Note: The surviving chicks from the above groups were challenged with 150,000 sporulated *E. tenella* oöcysts fourteen days after the original infection. There were some slight bloody droppings in group 1 A. The remaining groups showed no evidence of recurrence of coccidiosis.
A = natural infection (contaminated outside pens); B = experimental infection (wire-bottomed cages).

chicks were put in the contaminated pens, small groups of chicks the same age and from the same hatch were experimentally infected with 150,000 sporulated *E. tenella* oöcysts and put in wire-bottomed cages. These groups were given the same drug solutions that were used in the larger pens. The surviving chicks in all pens and cages were challenged fourteen days later with 150,000 oöcysts.

Table 3 shows the results following seven days' treatment under both natural and experimental conditions of exposure. Under natural exposure, there were no deaths in groups 1, 2, 3, and 4 during the week the drugs were given, but in group 5, the control group, there was a 50 per cent mortality from acute coccidiosis, with the rest of the controls showing severe clinical symptoms of coccidiosis. On the third day after removal of the drugs, there were hemorrhagic cecal evacuations in the group given sulfaguanidine and 1 death from acute coccidiosis in the sodium sulfamethazine group. The following day, all groups showed extensive hemorrhagic cecal evacuation; 1 chick died in the sodium sulfamethazine group and 3 in the sulfaguanidine group from acute coccidiosis.

The chicks in groups 1, 2, and 3, experimentally infected and kept in wire-bottomed cages, showed no evidence of coccidiosis. All chicks in group 4 showed clinical evidence of severe coccidiosis, although none died. All chicks in the control groups died from acute coccidiosis.

The surviving chicks in all groups were challenged fourteen days after the original infection with 150,000 freshly sporulated *E. tenella* oöcysts. There were hemorrhagic cecal evacuations in group 1A but none in the remaining groups. The controls used to test the potency of the challenging oöcysts showed a 73 per cent mortality. All the chicks, after the challenging inoculation, were kept one month in clean rat-proof pens but showed no further evidence of coccidiosis.

Experiment 4.—Three groups of 3-week-old and one group of 4-week-old New Hampshire chicks were put in naturally contaminated outdoor pens. Two of the 3-week-old and the 4-week-old group were given sulfaquinoxaline in the feed, and the remaining group of 3-week-old chicks was used as controls. The controls, in groups of 5, were moved systematically around the larger pens so that they covered the entire ground and had access to any oöcysts in the yard. Another group of 3-week-old chicks was kept coccidia-free in a battery brooder to be used as controls in the weekly tests. In groups 1 and 2, the drugs under test were given to the chicks as soon as they were placed in the contaminated pens. In group 3, the chicks were left in the contaminated pens four days before treatment was started. The sulfaquinoxaline was given in the feed four days, then omitted four days; this procedure was followed for five weeks. Each week, groups of chicks from the contaminated pens, along with coccidia-free controls, were challenged with 150,000 sporulated *E. tenalla* oöcysts. This was to test resistance developed to coccidiosis. Chicks taken from the exposure pens after the first, third,

TABLE 4—Results from Treatment of Chickens with Sulfaquinoxaline for Intervals of Four Days While Being Held in Naturally Eimeria Tenella Contaminated Outdoor Pens

Group	Sulfaquinoxaline in feed	Age—weeks	No. of chicks	Small groups challenged weekly with 150,000 sporulatel *E. tenella* oöcysts to test resistance developed to coccidiosis										Total died acute coccidiosis
				First[b] week		Second week		Third[b] week		Fourth week		Fifth[b] week		
				Evidence cecal hemorrhage	No evidence cecal hemorrhage	Evidence cecal hemorrhage	No evidence cecal hemorrhage	Evidence cecal hemorrhage	No evidence cecal hemorrhage	Evidence cecal hemorrhage	No evidence cecal hemorrhage	Evidence cecal hemorrhage	No evidence cecal hemorrhage	
1	0.05%	3	50	7[a]	3	3	7	1	9	0	10	0	10	1
2	0.1%	3	24	1[a]	4	2	8	1	3	1	4			1
3	0.1%	4	25	4[a]	1	2	3	1[c]		0	11			4
4	Susceptibility controls	3	20	0	5	0	8	0	5					2
5	Coccidia-free controls	3	50	10 / 8[d]	0	5 / 3[d]	0	10 / 7[d]	0	10 / 7[d]	0	15 / 9[d]	0	34

Note: Groups 1 and 2 given drug as soon as chicks put in pen fed drug four days and omitted four days; procedure for five weeks. Group 3 given drug after chicks were in pen four days; then drug fed four days and omitted four days; procedure for four weeks. Group 4 controls of 5 chicks in small movable pens in larger pens. Group 5 controls raised coccidia-free in batteries. Used in weekly tests to test potency of *E. tenella* oöcysts used in challenging test.

[a] = 1 dead in each from acute coccidiosis; [b] = Chicks in first, third, and fifth week tests held one week in wire-bottomed cages before being challenged with dose of *E. tenella* oöcysts; [c] = Died emaciation coccidiosis; [d] = Died acute coccidiosis.

and fifth week were held in wire-bottomed cages one week before being challenged.

During the first week, hemorrhagic cecal evacuations were observed in both the contaminated and control pens. Three deaths occurred from acute coccidiosis in group 3 during the first week (table 4), and 1 death each in the control pens of groups 2 and 3.

After the first week of treatment, a group of chicks was removed from each of the contaminated and control pens and held one week in wire-bottomed cages before being given oöcysts. These chicks along with coccidia-free controls from group 5 were challenged with 150,000 freshly sporulated *E. tenella* oöcysts. One chick in group 1 died during the first weekly test from acute coccidiosis after the challenging dose, and 6 others showed clinical and postmortem evidence of recurrence of coccidiosis. In group 2, 1 chick in the holding pen died from acute coccidiosis during the first week, before the challenging dose was given. One death resulted from acute coccidiosis during the first weekly test in group 3, and 3 chicks showed distinct clinical and postmortem evidence of coccidiosis. The controls from group 4, which had been in the contaminated pens, showed neither clinical nor postmorten hemorrhagic cecal changes, in any of the weekly tests.

Chicks in the second weekly test were taken directly from the pens and given the challenging dose. They showed more resistance after being in the contaminated pens for two weeks than did those held in the pens only one week (table 4).

Chicks removed from the pens after the third week were held in wire-bottomed cages for one week before being inoculated with oöcysts. They showed quite a resistance to the challenging dose, but there were still some clinical symptoms and postmortem evidence of cecal hemorrhages.

In the fourth weekly test, chicks were taken directly from pens and challenged with sporulated oöcysts; only 1 chick showed evidence of cecal hemorrhage.

In the fifth weekly test, the remaining group was held in wire-bottomed cages for one week before being challenged. None of these chicks showed cecal hemorrhage on postmortem examination.

All the controls used in the weekly tests to determine the infectivity of *E. tenella* oöcysts showed severe clinical and postmortem evidence of cecal hemorrhage, with a 68 per cent mortality from acute cecal coccidiosis (table 4).

Some of the chicks in each group, although not showing cecal hemorrhage on postmortem examination, did have old necrotic, cecal cores. In group 1, 4 chicks had small and 3 had medium-sized cecal cores, and 1 had a very large, necrotic core. In group 2, 5 chicks had small cecal cores, while in group 3, 1 chick had a small cecal core. The chicks with small cecal cores showed little change in weight from those that had no cecal cores, whereas those having medium-sized cecal cores did show weight losses, and 1 with a large, necrotic core was emaciated. The chick that died in the third week in pen 3 had very large, necrotic, cecal cores and was emaciated.

Experiment 5.—The effects of medicated feed on weight gains were tested with 120 New Hampshire 1-day-old chicks. Chicks were weighed and so arranged that each group had approximately the same type chick according to weight and sex, so that the average weight of the groups was the same. Four groups were used: Group 1 was given 0.0125 per cent sulfaquinoxaline in the feed continuously. Group 2 was given 0.05 per cent and group 3 given 0.1 per cent sulfaquinoxaline for four days, then the drug was omitted for four days. This intermittent procedure was followed for seven weeks. Group 4 was given feed with no drug. The chicks were weighed every twelve days. All groups were in coccidia-free, wire-bottomed batteries.

Table 5 shows the comparative, average weight of chicks fed various concentrations of sulfaquinoxaline in the feed. This feeding program was carried on for seven weeks. Groups 1, 2, and 4 showed approximately the same average weight gains, while group 3 showed a little loss in weight. One chick in group 2, killed when 48 days old, had an obstruction in the intestinal tract. There was

TABLE 5—Comparative Average Weights of New Hampshire Chicks Fed Various Concentrations of Sulfaquinoxaline in the Feed for Seven Weeks

Group	Concentration sulfaquinoxaline in feed	No. chicks	Ave. wt. (lb.) 21 days	33 days	45 days	57 days	70 days
1	0.0125%	30	.46	.90	1.38	1.97	2.74
2	0.05%	30	.46	.84	1.26	1.89	2.62
3	0.1%	30	.46	.80	1.20	1.69	2.39
4	Controls	30	.46	.88	1.36	1.95	2.64

Note: Chicks started on medicated feed at 21 days old. Group 1 fed medicated feed continuously; groups 2 and 3 fed drug in feed four days, omitted four days, procedure for seven weeks. All groups were in coccidia-free, wire-bottomed batteries.

little variation in the amount of feed consumed by the different groups, with group 3 taking slightly less feed than the others.

DISCUSSION

There are many advantages in the use of drugs to protect chicks against cecal coccidiosis while developing immunity, but there are also some disadvantages.

When infection is heavy, many chicks develop large cecal cores and other tissue changes which later result in losses or unthriftiness. The ingestion of sporulated oöcysts from the ground, as well as the consumption of the medicated feed or water, varies in a group of chicks. The infectivity of the same yard or pen varies greatly depending on many factors such as weather, output of oöcysts, and the availability of oöcysts to the chicks. The weight losses caused by the use of some drugs is an economic factor. Different groups of chicks also show variations in the protection from the same drug.

The medicated drinking water is not always readily taken by the chicks. It was observed that chicks in outside pens would drink the medicated water only if no other water were available. Following heavy rain, chicks would drink from puddles of water, or if the rain were light, they would ingest soil moistened by the drippings from the roof. This increased the intake of oöcysts and lessened the intake of the drug.

SUMMARY

Immunity tests were conducted with young chicks given drugs that previously had been tested and found to have some value in the treatment or prevention of cecal coccidiosis.

1) Chicks 4 weeks old were inoculated with large doses of *Eimeria tenella* oöcysts. Groups of chicks surviving this inoculation were later challenged to test the resistance developed. The tests were made at weekly intervals for a period of ten weeks, beginning two weeks following the original inoculation. A different group of survivor chicks was used for each test. All of the chicks that were challenged after the first inoculation were immune to further attacks.

2) Chicks were inoculated with large doses of *E. tenella* and given sodium sulfamerazine, sodium sulfamethazine, sulfaquinoxaline, and sodium borate for seven days. There were death losses from acute coccidiosis in the groups given sodium sulfamethazine and sodium borate. In the sodium sulfamerazine group, there were clinical symptoms of cecal coccidiosis but no deaths. The chicks in the sulfaquinoxaline group were not clinically sick and made the best weight gains. The survivors challenged four weeks later were immune.

3) Chicks given sodium sulfamerazine, sulfaquinoxaline, and sodium borate after the first symptoms of the disease appeared suffered losses in all groups. The sulfaquinoxaline group gave the best results. The survivors challenged four weeks later were immune.

4) Chicks, exposed both naturally and artificially, were treated for seven consecutive days with sodium sulfamethazine, sodium sulfamerazine, sodium borate, and sulfaguanidine. During this period of treatment, there were no losses from coccidiosis. After the treatment was discontinued in the naturally exposed group given sulfamethazine and sulfaguanidine, death losses ocurred within four days. There were no losses in the other groups. The survivors were immune to a challenging dose of oöcysts fourteen days later.

5) Immunity tests were conducted weekly on chicks under natural exposure conditions and given sulfaquinoxaline in the feed. Complete immunity in all groups was not observed until the fifth week. The controls, under similar exposure but not given the drug, were immune after the first week.

6) Three-week-old chicks given 0.0125 per cent and 0.05 per cent sulfaquinoxaline in the feed for seven weeks showed approximately the same weight gains. In the group given 0.1 per cent sulfaquinoxaline, there was some loss in weight and a slightly reduced feed consumption.

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Anthelmintic Activity of Sodium Fluoride in Chickens

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THIS INVESTIGATION was initiated in view of the reported excellent performance of sodium fluoride as an anthelmintic against large roundworms in swine, the numerous inquiries regarding the feasibility of employing the drug as an ascaricide for poultry, and scarcity of data on the subject in question.

At the time this investigation was begun, the only published report as to the efficacy of sodium fluoride as an anthelmintic in chickens was that of Habermann, *et al.*[1] These investigators reported that a ration containing 1.5 per cent of the drug given for ten consecutive days was devoid of anthelmintic action in 2 chickens. Later, Enzie and Jaquette[2] reported on the unsuitability of sodium fluoride as an anthelmintic for chickens. They reported on its activity against both *Ascaridia galli* and *Heterakis gallinae* in 27 mature birds. The drug was given both in capsule form and in the feed. Doses by capsule were 0.1, 0.2, and 0.3 Gm. per pound of the body weight (one treatment) and 0.5, 0.75, and 1.0 per cent in the feed for three days. They found the drug ineffective against heterakids at all levels. Against ascarids, the effective doses were too toxic by either method of medication to preclude any practical anthelmintic use of sodium fluoride in chickens.

METHODS

The first step in the present study was to give each of a group of 5 uninfected birds (average weight, 1¼ lb.) single doses of varied amounts of the drug to determine the tolerated dose of sodium fluoride. Birds were observed at two-hour intervals for the first few hours following administration of the drug and any symptoms were noted. Those noticeably affected by the drug were posted and examined for lesions.

The tests reported herein on the ascaricidal activity of the drug were conducted on 12 immature New Hampshires and White Leghorns and 10 White Leghorn hens. The New Hampshire chickens weighed an average of 2 lb. each in group 1 and White Leghorns of groups 2 and 3 weighed an average of 2¼ lb. each. They were previously given equal doses of an undetermined number of embryonated eggs of the large roundworm, *A. galli*. They were confined to batteries at all times and droppings examined three or four days prior to the treatment in order to check on the rate of spontaneous eliminations of the worms. Each of the 4 birds in group 1 received single doses of 0.01, 0.1, 0.25, and 0.5 Gm. of the drug by pipette. The drug was administered to group 2 (4 birds) for one day at the rate of 1 per cent of the feed and to group 3 (4 birds) for one day at the rate of 2 per cent of the feed. The droppings were collected and examined for parasites for three days following treatment in the first group and for five and four days, respectively, for groups 2 and 3. At the end of each test period, birds were autopsied for parasites and pathologic lesions noted.

The hens in groups 4, 5, and 6 were removed from a laying house and placed in individual compartments where they were kept for several days prior to the beginning of this study. The droppings were saved and screened for two to four days prior to treatment, in order to note spontaneous elimination of worms. Group 4 was then treated for three days with 1 per cent sodium fluoride in the feed; group 5 was given single doses of 0.3 Gm. (0.1 Gm./lb. body weight) of the drug by pipette; and group 6 was fed 0.5 per cent sodium fluoride in the feed for five days. All 10 hens had natural infestations of the large roundworm, *A. galli*, and the cecal worm, *H. gallinae*. All birds in groups 4 and 5, and 1 in group 6 had moderate infestations of the intestinal threadworm, *Capillaria columbae*.

RESULTS

Results of the determination of sodium fluoride tolerance were as follows: The drug exhibited pronounced toxic effects on birds receiving 0.5 Gm. and 1.0 Gm. each. The bird receiving 1.0 Gm. died within five hours following administration of the drug.

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Technical assistance was given by Mr. William Walsh, a senior in the School of Agriculture, Alabama Polytechnic Institute, Auburn.

The bird receiving 0.5 Gm. lost appetite; the comb paled; and the bird appeared inactive and depressed for three days, gradually improving until it appeared normal four days following administration of the drug. The bird receiving 0.1 Gm. exhibited mild ill effects for a few hours after administration of the drug, but appeared normal the following day. Birds receiving 0.05 and 0.01 Gm. each showed no ill effects of the drug. Symptoms and pathologic findings at autopsy of those receiving 1.0 and 0.5 Gm. were essentially the same as reported previously.[2] Results indicate that 0.05 and 0.01 Gm. of the drug were well tolerated.

Results of trials testing the efficacy of the drug are given in table 1. Treatment of ascarid-infested, immature chickens with single doses of sodium fluoride ranging from 0.01 to 0.5 Gm. resulted in low elimination of worms, when compared with the performance of well-known ascaricides. However, the bird receiving 0.1 Gm. eliminated 93 per cent of its ascarids. Sodium fluoride administered in the feed at 1 per cent (0.25 Gm. each) and 2 per cent (0.4 Gm. each) to groups 2 and 3, respectively, for twenty-four hours resulted in negligible elimination of worms. No worms were eliminated during the three-day observation period by groups 1 and 3 prior to treatment, whereas 3 ascarids were eliminated by group 2 during the pretreatment period. Birds in group 1 receiving 0.25 Gm. and 0.5 Gm. of the drug showed symptoms of depression for one and two days, respectively. Birds in group 2 exhibited no ill effects of the drug, whereas those in group 3 ate approximately 4/5 of their normal ration and showed mild depression for one day.

The 4 hens in group 4, receiving 1 per cent sodium fluoride in the feed for three days, eliminated 89 per cent of their ascarids. However, they consumed only ⅓ to ¼ of their normal rations. One bird, that had been in production, promptly quit laying. These birds lost an average of 190 Gm. each during the treatment period. They appeared depressed; their droppings were sparse, mucoid, watery, and consisted mostly of urates. Hens in group 6, receiving 0.5 per cent sodium fluoride in the feed for five days, eliminated an average of 65 per cent of their ascarids. They ate more feed than those in group 4 but failed to consume as much as they would normally eat. All hens of group 6 were in production prior to treatment but failed to lay any eggs after the third day of medication. They appeared mildly depressed and lost an average of 170 Gm. each during the treatment period. The 2 hens in group 5 that received 0.3 Gm. of the drug each had a loss of appetite and appeared depressed on the day the drug was administered, but they recovered the following day. They eliminated only 33 per cent of their ascarids. As shown in table 1,

TABLE I—Data on Action of Sodium Fluoride as an Anthelmintic in Chickens

Group	No. of birds	Dose	Method of Administration	Parasites			Efficacy (%)
					Removed	Found at autopsy	
1	1	0.01 Gm.	Pipette	A.	0	33	0
	1	0.10 Gm.	Pipette	A.	44	3	93
	1	0.25 Gm.	Pipette	A.	19	14	58
	1	0.50 Gm.	Pipette	A.	10	13	43
2	4	1.0%	In feed, 1 day	A.	1	38	2
3	4	2.0%	In feed, 1 day	A.	2	36	5
4	4	1.0%	In feed, 3 days	H.	38	322	11
				A.	195	24	89
				C.	5	357	1
5	2	0.1 Gm./ lb. body wt.	Pipette	H.	93	482	13
				A.	10	20	33
				C.	1	102	1
6	4	0.5%	In feed, 5 days	H.	?	Many	?
				A.	13	7	65
				C.	?	Few in one	?

A. = Ascaridia; H. = Heterakis; C. = Capillaria.

the number of heterakids and capillarids eliminated by groups 4, 5, and 6 during and following medication was negligible. Groups 4 and 5 eliminated a total of 3 ascarids during the two-day pretreatment period and group 6 eliminated 2 worms. Hens in groups 4 and 5 were held and droppings screened for five and eight days following treatment. Those in group 6 were held and droppings screened for three days following treatment. They were then autopsied, all intestinal contents screened, and the remaining worms counted.

CONCLUSIONS

Although the experiments described are limited, the data indicate that sodium fluoride, at nearly effective levels for ascarid elimination, is toxic to mature birds and causes them to go out of production. It is very inactive against heterakids and has practically no activity against *Capillaria columbae*. At tolerated levels, it is also ineffective against Ascaridia in growing chickens. The data gathered, in general, indicate that doses of sodium fluoride at tolerated levels are so ineffective as to preclude its use as a practical anthelmintic in chickens.

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Immunization of Chickens Against Fowl Plague

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NUMEROUS attempts have been made to reduce the usual high virulence of the virus of fowl plague to such degree that exposure of chickens to it would result in immunity. The procedures that were employed involved treatment of the virus by physical and chemical means, exposure of chickens to infective yet sublethal doses of the virus or to small lethal doses simultaneous with, or prior to, the injection of large amounts of specific antiserum, subjection of the virus *in vitro* and *in vivo* to specific antibodies and other immunobiologic factors, and subjection of the virus to culture in explants of avian tissues and to passage in the tissues of alien hosts.

The treatment of tissues of plague-infected chickens by various physical and chemical means provoked either no immunity or low and irregular resistance. Thus, treatment of the tissues by heat, desiccation, chloroform, saponin, ether, phenol, and glycerine, either alone or in combinations, have been described. The reports have been adequately reviewed by Gerlach[1] and Hallauer.[2] Staub[3] was the first to employ formalin, and the procedure was considered on limited data to be useful in vaccine production. The procedure was likewise found satisfactory by Morcos,[4] whose studies were conducted apparently without knowledge of the previous use of formalin on the plague virus. While Staub's observation was not confirmed in several instances,[5, 6, 7] the studies of Plotz,[8] Hallauer,[9] and Nakamura, *et al.*[10] appraised the procedure as having merit. Attempts to alter the virus suitably by aluminum hydroxide[7] and by pressure[11] met with failure.

Investigations on the development of immunizing procedures, other than by primary treatment of the virus with physical and chemical agents, were first recorded on the use of small, or sublethal, amounts of the virus (possibly the absence) by Belfanti and Ascoli.[12] The unsuitable nature of the procedure was later confirmed.[13] Mixtures of virus and of sufficient antiserum to destroy the infectivity of the virus were found to lack immunogenic properties.[2, 14, 15] Successful immunization was effected by injection of chickens with antiserum one day previous to the inoculation with the virus,[15] although with comparable quantities of virus and serum Hallauer[2] was unable to confirm the observation. However, the simultaneous injection of large amounts of serum and of small amounts of virus was shown by Manninger[16] to be of some, but limited, value. In this regard, Hallauer[2, 17] demonstrated that a strong immunity resulted from simultaneous treatment with serum and virus only when large amounts of serum (over 20 cc.) were used and both materials were injected intravenously; the chickens that were treated in this manner showed, however, a transient illness. Three chickens that had been immunized in this manner were later subjected to an intracerebral injection of virus, and tests of brain, liver, spleen, and kidney revealed that liver and spleen collected at twelve and twenty-four hours after the challenge exposure possessed immunogenic properties. Hallauer[17] demonstrated further, in apparent confirmation of certain studies by Erdman,[18] that while the fowl plague virus may multiply in tissue culture of chicken brain, other tissues, particularly liver of adult chickens and chicken embryos, rendered the virus avirulent and immunogenic.

This work was done as a part of a research project conducted under the direction of a War Department commission consisting of: Brigadier General R. A. Kelser, U. S. Army; Dr. R. E. Dyer, U. S. Public Health Service; Dr. H. W. Schoening, Pathological Division, Bureau of Animal Industry, U. S. Department of Agriculture; and Dr. E. B. Fred, University of Wisconsin.

Special research assistant, Dept. of Comparative Pathology and Tropical Medicine, Harvard Medical School, now associate pathologist, Purdue U., Agric. Expt. Sta. (Moses); special research associate, Dept. of Comparative Pathology and Tropical Medicine, Harvard Medical School, on leave, U.S.D.A., Agric. Res. Administration, BAI, now at the Dept. of Vet. Sci., U. of Wisconsin (Brandly); associate professor, Wellesley College (Jones); and special research assistant, Dept. of Comparative Pathology and Tropical Medicine, Harvard Medical School, on part time leave, U. of Connecticut and Storrs Agric. Expt. Sta. (Jungherr).

The procedure was seriously limited, however, by inability to standardize the procedure in a manner that was readily reproducible.

All studies that have been directed primarily toward either alteration of the virus by passage in geese, and subsequent desiccation of the nerve tissues,[19] or modification of the virus by passage in mice and other small mammalian hosts[20, 21] and in cultures of embryonic fowl brain[22] failed to provide satisfactory material for immunizing purposes.

The products of lethally infected embryonating chicken eggs were employed first for the production of vaccines by Traub.[23] The amount of virus obtainable by egg cultivation was shown by him as well as by others[24, 25, 26] to be higher and more uniform than that in chicken or other tissues. The results obtained with a formalinized vaccine and the Rostock strain of virus were very encouraging.

This paper is concerned with a description of investigations on immunization of chickens with egg-propagated, fowl plague (FP) virus of the Dutch East Indies (D.E.I.) strain and of variant strains of virus isolated from it. The studies were begun in July, 1943, and were continued over a thirty-month period.

PREPARATION OF VACCINES

At the outset of this work, information was available to the effect that FP virus could be propagated in embryonating chicken eggs and that higher and more uniform concentrations of virus were attainable thereby than in the tissues of infected chickens. Therefore, evidence was sought first of the value of egg-propagated virus for purposes of vaccination.

The merit that had been demonstrated by the earlier workers for formalin in the preparation of vaccines from tissues of FP-infected chickens, together with its known usefulness in the production of vaccines with the egg-cultivated virus of equine encephalomyelitis,[27] supported the initial choice of this agent for purposes of inactivating the egg-propagated FP virus. Ultraviolet (u.v.) light was subsequently used in comparative trials with formalin as a means of destroying the virus infectivity and still maintaining its immunogenicity. Studies were conducted to determine the adjuvant effect of several materials on the immunogenic properties of the treated components of lethally infected eggs. Assessments were made on the effect of different pH values and of storage on the vaccines.

Strains of FP Virus Used. — The Dutch East Indies (D.E.I.) strain of virus was obtained for, and employed throughout, this study. In addition, strains of virus that were isolated from chicken embryos and were of low virulence for chickens were considered. The variant strains of the D.E.I. virus referred to in this study are designated as variant 4395 and variant 19493. These same designations were used in other articles where the origins and certain characteristics of the variants as well as of the D.E.I. strain were presented.[21, 28, 29]

Propagation of Fowl Plague Virus.— Throughout the course of the studies, attention was given to factors that might influence the procurement of maximal amounts of suitable virus. Only eggs from White Leghorn stock of a single farm were used. All embryonating eggs employed for the propagation of virus that was ultimately related in any way to the preparation of the vaccines to be described or to serologic assessments of their value were incubated at 37 to 38.5 C. for ten to twelve days prior to, and following, test inoculations. The incubators were of the forced-draft type, and the humidity was controlled at levels between 70 and 75 per cent.

D.E.I. Strain.—Following the initial establishment of infection of comparable course by the D.E.I. virus in embryonating eggs inoculated by various routes, it was observed that high concentrations of virus accompanied by death of the embryo[21, 28] were demonstrable in the allantoamnionic (a.a.) fluids following injection of the virus into the allantoic chamber (a.c.). The amounts of virus were from 20×10^8 to 20×10^9 embryo m.l.d.'s of virus per cubic centimeter of a.a. fluid, and were comparable to those obtained in both the a.a. fluids and the embryo itself following suprachorioallantoic (ca.m.) inoculation. The attainment of this concentration of virus was unaltered during ten early serial passages of the D.E.I. virus by the a.c. route, in which passage series of the virus were maintained by inoculation of groups of eggs with undiluted, 10^{-3} or 10^{-6} dilutions of a.a. fluids from the previous passage. No alteration of virus titers was observed in the a.a. fluids during 25 further serial passages of the virus by the a.c. route when employing 100 m.l.d.'s of virus as inoculums.

Comparative data were obtained on the attainable titer and the rapidity of its appearance in two components of the embryonating egg following the deposition of small amounts of virus, usually 100 m.l.d.'s, by the a.c. intravenous (i.v.) and ca.m. routes. In concurrent studies involving the a.c. and i.v.

routes, the condition of the eggs was noted, and groups of 2 or 3 of them were removed for study at three-hour intervals postinoculation. The embryos that were inoculated by the i.v. route were noticeably abnormal in movement and color as early as fifteen hours postinjection, while the embryos inoculated by the ca.m. route showed the first gross evidence of abnormality six hours later. Titrations were made of the embryo-infectivity of a.a. fluids and of supernates of minced embryos. At one thousand fold levels of dilution, virus was first demonstrable in 0.05-cc. amounts of the lowest dilution of both a.a. fluids and material from the embryo proper at three hours postinoculation. Within twelve to fifteen hours later, virus appeared to be consistently close to the maximal titers obtainable. Continuation of incubation until death of the embryos, twenty-one to twenty-four hours postinoculation for the i.v. inoculates and twenty-seven to thirty hours for the a.c. inoculates, had no demonstrable effect on the virus titers. In other tests, the survival time of the embryos and the rate of appearance of the virus in the a.a. fluids of eggs inoculated on the ca.m. was shown to be comparable to the conditions established for a.c. inoculation. Data that indicate or show the rapidity of the increase of virus in a.a. fluids and/or in the embryos are shown in table 1 and figure 1.

TABLE 1—Effect of Incubation Continued After Death of Embryos on Virus Titer and pH of Allantoamnionic and Embryo Fluids

Hours in incubator		Log of virus titer/0.05 cc.		pH	
After inoculation	After death	A.a. fluid	Embryo fluid	A.a. fluid	Embryo fluid
24		8	7	8.00	6.88
28	0	8	7	7.78	6.67
32	4	9	8	7.90	6.64
36	8	7	7	7.83	6.66
40	12	6	7	7.70	6.64
44	16	8	7	7.55	6.70

Further testing to determine the distribution of virus in other components of lethally infected embryos which were inoculated when 12 days old by the a.c. route showed apparent wide distribution and considerable uniformity in the concentrations of the virus. The yolk, which comprised the greatest bulk of the individual components, contained the smallest amounts of virus of any of the portions that were included in the vaccines (albumen was always discarded because of its physical character). The virus titer of yolk was found not to exceed 20 x 10^5 m.l.d.'s per cubic centimeter, and, in some tests, was one hundred fold lower. Composites of washed yolk sacs from groups of 2 or 3 eggs each contained amounts of virus similar to those previously mentioned for the a.a. fluids and embryo bodies. Similarly high titers were found in composites of yolk sacs and contents, of chorioallantoic and amnionic membranes, and of the total usable contents of the eggs, without washing of any parts, from groups containing 100 or more eggs.

Attention was given to the possible effect on uniformity in virus production that might result from incubation of groups of infected eggs until 50 per cent were moribund or dead. In this regard, the data shown in table 1 were obtained for eggs that were embryonated twelve days prior to a.c. inoculation with 100 m.l.d.'s of virus. With the two unexplained variations that occurred in the virus titers of the a.a. fluids of eggs removed at four and twelve hours after death, the content of virus in the fluids and in the extracts of embryos at four hours prior to death and up to sixteen hours after death was comparable to the results obtained for materials removed from the eggs at the approximate time of death. The pH values of the materials appeared not to constitute an alteration deleterious to the virus.

TABLE 2—Rate of Inactivation of Fowl Plague Virus by Ultraviolet Light

Type of light, in wave lengths					
		1,600 to 1,800 Å			
2,537 Å*		Trial No. 1		Trial No. 2	
Exposure time, (min.)	Embryo infectivity-titer**	Exposure time, (sec.)	Embryo infectivity-titer**	Exposure time, (sec.)	Embryo infectivity-titer**
0	8	0	8	0	7
5	7	0.048	8	0.52	2
15	6	0.24	4	0.72	0.1
25	4	0.56	2	1.12	0.1
35	1	0.78	1	3.0	0.1
45	0	1.10	0	3.8	0

*This test is one of numerous trials conducted as explained in the text.

**The titration was conducted by a.c. inoculation of embryonating eggs with 0.1 cc. of the undiluted or diluted virus materials; the titrations are expressed as the reciprocal of the log of the dilution that was lethal for embryos.

The effect of postinoculation incubation of eggs at temperatures above and below the usual was considered as being of possible importance. Ten a.c. passages of the virus were, therefore, conducted simultaneously at 34 and 40 C., with the usual temperature for control purposes (37.5 C.). Less than 100 m.l.d.'s of virus in the a.a. fluids were used as inoculums. The results showed that, whereas incubation at 40 C. was associated with a slight average decrease in survival times of the embryos, there occurred at 34 C. an approximately 75 per cent increase in the average survival period in comparison to the ones incubated at 37 C. The embryo-infectivity titers of virus in the a.a. fluids were essentially the same in all cases.[5]

Information obtained early in the course of the studies indicated that eggs embryonated ten to twelve days prior to inoculation yielded the most suitable egg components for use in the preparation of vaccines.[26] With shorter

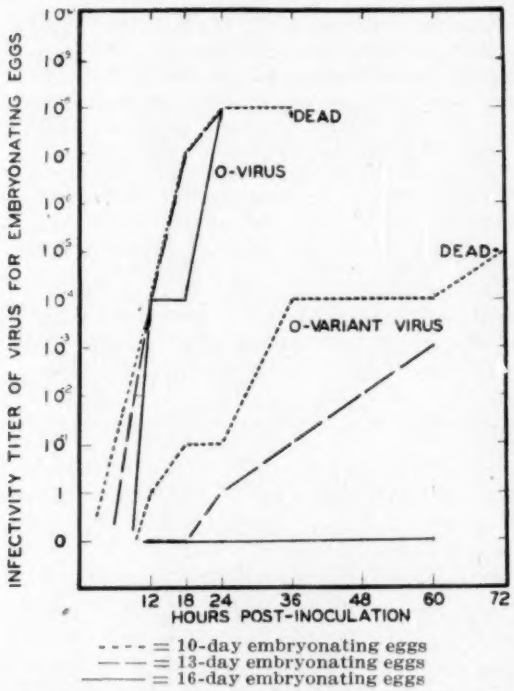

Fig. 1—Infectivity titers of D.E.I. (O) and of variant 4395 (O variant) in allantoamnionic fluids of embryonating eggs at intervals postinoculation.

preincubation periods, the bulk of the a.a. fluids and of the embryos was markedly smaller; while in eggs that had been incubated fourteen days or longer, the yield of a.a. fluids was again reduced, and the feathers and the fibrous and osseus tissues of the embryos which were present at that age rendered the material unsuitable for incorporation in vaccines. The essentially similar rate of appearance of the virus in the a.a. fluids of 10- and 13-day-old embryos and the possible transient retardation of its development in 16-day-old eggs following a.c. inoculation of 100 m.l.d.'s of virus are shown in figure 1.

As a possible added assurance of maintenance of suitable stock virus for use in vaccine production, an i.v. series of virus passage was initiated and carried for 31 consecutive transfers in embryos. In this procedure, 15-day-old embryonating eggs were injected by way of the allantoic vein with 0.05 cc. of a dilution of the plasma from the previous group of embryos. This inoculum usually contained 10 or 100 m.l.d.'s of virus, which was determined in each case by injection of other groups of embryos with tenfold greater dilutions of the plasma to include the 10^{-9} dilution. Embryos that were in the regular series or line of passages were exsanguinated approximately twenty-four hours postinoculation, or just prior to death, by withdrawal of the blood directly from a vein or artery in the ca.m. Heparin was used as anticoagulant in the early passages but was discontinued later upon observation of the failure of the infected blood to coagulate. The virus maintained all of its original infectivity for embryos throughout the passages. Its infectivity for chickens, like that of the a.c. line previously described, was not perceptibly altered when tested at the conclusion of every five passages.

All vaccines hereafter described were prepared from embryonating eggs that were infected at 10 to 12 days of age by a.c. inoculation.

Variant Strains 4395 and 19493.—The isolation of variant strains of FP virus from embryos inoculated with infectivity-end-point dilutions of the D.E.I. virus and certain characteristics of the variants have been described in previous papers.[21,25,29] The relative avirulence and immunogenicity of the variants for chickens pointed to their possible value as immunizing agents for use in the living state either alone or following the injection of killed virus vaccines.

The variant viruses were capable of producing lethal infection in embryos by all pro-

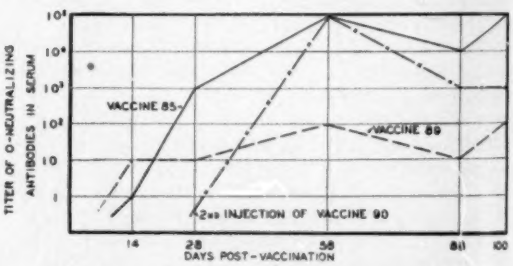

Fig. 2—Serum antibody titers for the D.E.I. virus (O-neutralizing antibodies) following a single injection of adjuvant (85) and of nonadjuvant (89) vaccines and of two injections of nonadjuvant vaccine (90).

cedures tested, that is following a.c., ca.m., and i.v. inoculation. The course of the disease that was produced in embryos was about twice as long as that produced by comparable amounts of the parent strain (*see* fig. 1). Unlike the D.E.I. virus, ten- to one hundred fold greater amounts of the variant viruses were required to produce lethal infection in 12-day-old eggs inoculated by the a.c. or ca.m. routes than by the i.v. route. Furthermore, as shown in figure 1, the rate of appearance in the a.a. fluids of one variant tested in this regard was significantly decreased following

a.c. injection of 13-day-old eggs in comparison to embryos three days younger. In 16-day-old eggs, multiplication of the virus was either completely suppressed or its viability was destroyed. The eggs were inoculated with 10^2 m.l.d. of virus. Amounts of virus that were 100 to 1,000 times greater than those required to kill the younger embryos were found, however, to infect lethally the 16-day-old eggs following a.c. injection.

The concentrations of the variant viruses in the a.a. fluids of lethally infected eggs were found to be quite uniform. The maximal titers demonstrable by a.c. inoculation of 10- to 13-day-old eggs were 20 x 10^4 or 20 x 10^5 m.l.d.'s per cubic centimeter (*see* fig. 1).

The 4395 and the 19493 variants were passed serially through 15 and 10 groups, respectively, of 10- to 12-day-old embryos. In each case, 10 to 100 m.l.d.'s (a.c.) of the variants in a.a. fluids were injected by the a.c. route. No alterations in their virulence for embryos or in the nature of the disease were observed during this time.

As a possible means of challenging further the stability of one of the variants (4395 strain), simultaneous serial passages were conducted in embryos that were incubated postinjection at 34, 37, and 40 C. The embryos were injected with 10 m.l.d's (by a.c. titration) of the virus, in a.a. fluids from the previous group, by the a.c. route. During nine passages at 34 C. and ten passages each at 37 and 40 C., no significant alterations were observed in the titers of virus that developed in the a.a. fluids. The essential nature of the disease was also unaltered, except that at 34 C. the embryos survived for an average period that was approximately 55 per cent longer than at 37 C., and at 40 C. there occurred a decrease in survival time of about 20 per cent.

All of the variants used for vaccination purposes were contained in a.a. fluids of lethally infected embryonating eggs inoculated by the a.c. route.

Inactivation of FP Virus in Infected Embryo Tissues.—By Formalin.—For the inactivation of the virus in chicken tissues, Staub[3] first recommended a 0.005 per cent concentration of formalin, but later he recommended[31] a level of 0.0015 to obtain more consistent results. Morcos[4] employed 0.001 per cent of formalin with satisfactory results. Hallauer,[9] however, added formalin to the extent of 0.25 per cent but suggested that greater amounts should not be used.

Because of the foregoing information and because of data on vaccines prepared with other egg-propagated viruses, the concentrations of formalin used in these studies were restricted to not more than 0.4 per cent. The maximal amount of formalin was commonly used to destroy the infectivity of the virus in the fixed tissues, or membranes, of the embryonated eggs, whereas 0.25 per cent was used for the treatment of the a.a. fluids and of the whole egg material, or of composites of the entire contents of the egg other than the albumen. The materials were stored at 6 to 10 C. following addition of the formalin and thorough shaking. Tests for the destruction of the infectivity were made in embryonating eggs, each of several eggs being injected by the a.c. route with 0.1 cc. of the material. Approximately 90 lots of embryonating egg components were prepared in this manner for use as vaccines.

The rapidity of inactivation of the D.E.I. virus in a.a. fluids of lethally infected eggs by 0.05, 0.1, 0.2, and 0.4 per cent concentrations of formalin was determined for the temperature of 6 C. All materials were still infective following five minutes of exposure. After the

TABLE 3—Influence of Age at Vaccination on the Development of Immunity to Fowl Plague as Determined by Challenge to FP Virus at 21 Days Post-vaccination

Experiment No.	Age of chicken	Results of challenge exposure*	
		Vaccinated** chickens No. survived/ No. exposed	Control chickens No. survived/ No. exposed
1	1 week	11/12	0/4
	1 month	10/12	0/4
	2 months	10/11	0/4
	Hens (1-4 yr.)	6/8	0/4
2	1 week	10/10	0/4
	1.3 months	11/11	0/4
	2.5 months	12/12	1/4
	3.5 months	8/8	0/2
	4.5 to 5.5 months	8/8	0/2
	Cockerels (1 year)	3/4	0/2
	Hens (1-4 yr.)	10/10	0/4

*The challenge exposure was given by intravenous injection of the virus in both experiments; in the first one of these, 10^3 or 10^5 m.l.d.'s were given to one half of each group, while in the other 10^2 or 10^4 m.l.d.'s were given to one half of each group save in the smaller groups of controls which were given the smaller dose.

**The composition of each cc. of the vaccines (the dosage injected) for each experiment follows: Experiment 1: formalinized (0.25%) a.a. fluid, 40 per cent, and embryo pulp, 20 per cent, containing 12 x 10^8 units of virus; Experiment 2: formalinized (0.25%) a.a. fluid, 30 per cent, and embryo pulp, 20 per cent, which were incorporated into a mixture of 10 per cent Falba, 40 per cent mineral oil and 140 mg./100 cc. *Mycobacterium butyricum*, containing 10^9 units of virus.

TABLE 4—Comparison of Immunogenicity of Formalinized D.E.I. and Variant Viruses

Virus			Assessment of immunity							
	Amount in a.a. fluid		Antibody titers, days post-vaccination						No. survived/No. exposed to D.E.I. virus, m.l.d.	
Name	cc.	Units	0	14	28	42	59	98	10	10^2
D.E.I.	10.0	2×10^{10}	0	10	10^3	10^4	10^3	10^4	2/2	2/2
	1.0	2×10^9	0	1	10	10^3	10^2	10	1/2	1/2
	0.1	2×10^8	0	1	1	0	10	0	0/2	0/2
	0.01	2×10^7	0	0	0	0	0	0	0/2	0/2
Variant 4395	10.0	2×10^8	0	0	0	0	0	10	1/1	1/2
	1.0	2×10^7	0	0	0	0	0	0	0/2	0/2
	0.1	2×10^6	0	0	0	0	0	0	0/2	0/2
	0.01	2×10^5	0	0	0	0	0	0	0/2	0/2
None			0	0	0	0	0	0	0/2	0/2

next test interval of six hours, the virus was noninfective in the materials that contained 0.4 and 0.2 per cent of formalin. The 0.1 per cent level required between eighteen and twenty-four hours to inactivate the virus, while the lowest concentration required more than thirty-one hours but less than 216 hours to destroy the infectivity.[27] Determinations with a glass electrode potentiometer of the pH of the materials after storage for forty days revealed that the specimen containing the smallest amount of formalin was only slightly less alkaline than the untreated portion of the a.a. fluid (pH 7.6), but that the increase in the concentration of formalin up to 0.4 per cent caused a progressive decrease to pH 6.44. Material with 0.2 per cent formalin, nearest the concentration (0.25) commonly used was reduced to pH 6.73. This value appeared to be within the range of maximal stability of the virus.

General information obtained for the 4395 variant virus showed that it was inactivated by formalin in a manner comparable to that of the parent, or D.E.I., strain.

By Ultraviolet Light.—The observation of Hodes, *et al.*[32] that the infectivity, but not the immunogenicity, of the rabies virus for mice was destroyed by energy consisting mainly of u.v. light of 2537 Å wave length suggested the possible usefulness of this means of inactivation of the FP virus.

A Hanovia unit, producing the type of light used by Hodes and his associates, was used to irradiate the D.E.I. strain of virus contained in a 35-cc. capacity quartz tube of 25 mm. inside diameter (I.D.) about which the tube emitting the light was coiled. Transparent a.a. fluids from lethally infected eggs were used in the tests. In preliminary tests, considerable irregularity was encountered in establishing the time required to inactivate the virus; variations between thirty-five and forty-five minutes were observed.[32] The typical rate of inactivation observed in these tests is shown in table 2. In subsequent trials, the unit was aërated, and the virus material was mechanically stirred in order to prevent heating of the virus more than a few degrees above room temperature and to facilitate more uniform exposure of the material. These alterations in techinque resulted in a reduction of the necessary period of irradiation to as low as twenty minutes. Material of this type was tested for immunizing properties in chickens in comparison with a portion of the same virus material that was inactivated with formalin.

The demonstration of an immunogenicity for the Hanovia-treated virus inferior to that of the formalinized material prompted the direction of further studies to the newly described procedure of Levinson, Oppenheimer, and associates.[34] This procedure utilized energy associated with wave lengths mainly of 1,600 to 1,800 Å and had yielded encouraging results in preparing vaccines with several viruses.[34] Studies with the D.E.I. virus were

TABLE 5—Influence of Vaccination with Inactivated Virus Followed by Intravenous (i.v.) Exposure to Living FP Virus on Antibody Titers and on Resistance to Intercranial (i.c.) Exposure to the Virus

Group No.	Time and kind of treatment prior to i.c. challenge		Antibody titers in relation to i.v. challenge		No./survived No./inoculated i.c. with virus, m.l.d.		
	Dead virus vaccine*	Living virus exposure	Before	After	10^5	10^4-10	1-0.1
1	91 days	21 days	10^2 to 10^4	10^6 to 10^8	14/15		
2	119 days	21 days			15/16		
3	103 days	none			0/4	1/16	
4	none	none			0/2	0/2	2/4

*The vaccine, injected intramuscularly, was No. 61 (pH 8) and was composed of 40 per cent a.a. fluid, 20 per cent minced embryo, 40 per cent buffer, and 0.25 per cent formalin and contained 20×10^8 virus units per cubic centimeter.

made possible by the courtesy of the authorities of the National Institute of Health, Bethesda, Md., where the exposure procedures were conducted by Dr. Karl Habel.

The virus material for these tests consisted of the supernate obtained by centrifugation of a mixture of 9 parts of a.a. fluid and 1 part of finely minced embryos from lethally infected eggs. The rates of inactivation of the virus obtained in two trials are shown in table 3. Tests for end points of virus infectivity by inoculation of chickens parenterally with 1 cc. of the undiluted virus materials gave results similar to those shown for embryos. The period of irradiation was in excess of 0.78 seconds in one trial and greater than 1.12 seconds in the other. The differences in the periods of irradiation required to kill the virus appeared attributable to loss of potency of the light source with continued use.

The Addition of Adjuvants. — Prior to August, 1944, the results obtained with representative types of 65 inactivated virus vaccines indicated a need for further practical means to enhance the immune response. The need was emphasized by the occurrence of too frequent lethal effect of the exposures used in challenging the resistance. The potential for greater response was shown by the results of repeated injections of the inactivated virus, of exposure to living virus following the injection of dead virus, and of injection alone of the living variant viruses. Attention was directed to procedures described recently by Friedewald[35] that resulted in the enhancement of the immunogenicity of influenza virus vaccines and involved the incorporation of paraffin or of sesame oil, or of a proprietary oily material possessing absorptive properties known as Falba,* and of killed acidfast bacteria. Consideration was given also to alumina gel, which had shown considerable promise in other viral vaccines, as mentioned in a previous report,[28] and, in particular, in a report[25] on FP that appeared coincident with our investigation.

Portions of basic vaccines as described in preceding sections were modified by the addition of Falba,* mineral oil, sesame oil, and killed acidfast bacteria, either alone or in various combinations, and of alumina gel.** The influence on the immunizing properties of the vaccines was studied with respect to different concentrations of the adjuvants and to variations in the order or means whereby the components were admixed. Vaccines containing Falba were compounded by two common procedures, namely, by incorporation

*Manufactured by Pfaltz and Bauer, Inc., New York.

**The product was designated H81, and was manufactured by the Aluminum Ore Company, East St. Louis.

TABLE 6—Immunogenicity of Various Amounts of Living Variants of FP Virus

Variant injected intravenously		Assessment of immunity				
				No. remained healthy/No. challenged i.v. with D.E.I. virus, m.l.d.		
No.	Embryo m.l.d.	Days post-vaccination	Log of antibody titer	10^2	10^4	10^5
4395	2×10^6	21	7	1/1*		
	2×10^5		5-6	4/4		
	2×10^4		6-7	2/2		
	2×10^3		5	1/1*		
	2×10^2		5-6	2/2		
	2×10		5-8	2/2		
	2		6-7	2/2		
	0		0	0/2		
19454	10^7	105	7		3/3	
	10^6		5		3/3	
	10^5		5		2/3	
	10^4		4		1/3	
	10^3		3		3/3	
	10^2		4		1/3	
	10		2, 2, 6		3/3	
	0.1		0, 0, 2		0/3	
	0		0		0/1	
4395	10^2	105	4-6			9/9

*Four chickens originally in these groups died of infection by the variant in the second and seventh days of the experiment.

with mortar and pestle of embryo material into the Falba (1 part absorbed 4 of a.a. fluid) and subsequent addition of mineral or sesame oils and by addition of the basic vaccine to a mixture of mineral oil and melted Falba in a mechanical mixer. One vaccine was prepared, also, to test the possible adjuvant effect of finely minced normal embryos. Aside from the influence on immunogenic properties described in subsequent sections, consideration was given to the effect of the adjuvants on the consistency of the vaccines and to undesirable tissue reactions in chickens. The principal observations made on the fowl plague vaccines or on concurrent studies of Newcastle disease [avian pneumoencephalitis] vaccines[36] included:

1) Concentrations of Falba greater than 5 to 7.5 per cent in conjunction with less than 20 to 25 per cent mineral oil produced vaccines that could be manipulated for injection only with considerable difficulty, and greater amounts of either of the materials produced severe or prolonged tissue reaction.

2) Killed, acidfast bacteria, of the species *Mycobacterium butyricum*, contributed to the local inflammatory response of the vaccines and provoked sensitization to avian tuberculin.

3) The alumina gel could be incorporated in concentrations as high as 50 per cent without causing either unsatisfactory consistency of the vaccines or objectionable changes in tissues when employed as described later.

Testing and Standardization.—In all tests involving immunogenicity of living D.E.I. virus or variant strains thereof, a titration of the virus content was made in embryonating eggs with the same virus materials that were employed as inoculums for chickens. The titrations of the variant strains were conducted by the route that gave the higher result, or, as mentioned previously, by the i.v. route of inoculation. Embryonating eggs were also used for the titration of infective virus in representative samples of all materials employed for the production of inactivated-virus vaccines. The number of m.l.d.'s of virus revealed by this test per quantity of test inoculum (usually .05 cc.) was considered as the number of virus units in a similar quantity of the material in which the infectivity of the virus had been destroyed. These figures were used to compute the total number of virus units in all vaccines.

The choice of the plan for assessing the merits of the vaccines depended on the objective and on other factors of the experiment and, in the later phases of the work, was influenced by earlier experience or the general nature of the resultant immunity. Either of two general plans was employed in most of the tests. In one procedure, the dosage of the vaccines was held constant, and the degree of challenge exposure was varied. The reverse of this was conducted in the other case. Dosages of the vaccines containing the maximal amounts of virus and one or more tenfold dilutions thereof were injected, and the subsequent challenge exposure with the D.E.I. virus was held constant. The former procedure was more useful in studies on the initial development of immunity, or that occurring within ten to twelve days postvaccination. In this particular case, the short period of time during which the animals were maintained was compensatory for the proportionately large number of animals required. The second of the two plans was considered better for use in comparative studies of the immunogenicity of (1) a.a. fluid and minced embryo material and (2) of vaccines with and without adjuvants.

In investigations of the immunizing properties of the viruses, cognizance was given to the possibility of provocation or enhancement of an initial immunity to the disease by the spread of living viruses among the vaccinated chickens. For this reason, all chickens used in tests on inactivated viruses were maintained in strictly isolated quarters where evidence of living virus was never detected. On the other hand, just prior to exposure to the living virus, the chickens were removed to other isolation quarters, where they were held until sacrificed for further study. Spread of the living viruses among chickens previously immunized to a substantial degree with inactivated virus was apparently of a low order or nonexistent. However, an exception to this occurred under conditions of gross simulated natural exposure to the D.E.I. strain. Injection of the living variant viruses into fully susceptible chickens was also associated with transmission to other groups in the experiment, but this appeared to be related to cannibalism. The addition of untreated or control chickens to the environment served for gauging such transmission.

Supplemental evidence on the degree of isolation of the experimental groups and on the immunogenicities of the vaccines was obtained by studies of serum collected before and after vaccination, as described in a subsequent section. The serum of stock chickens, or those without any history of known possible contact with the virus, and those vaccinated less than eight to ten days previously, failed regularly to alter the end point infectivity titer of the D.E.I. virus. However, the test was considered to be of considerable merit in estimating the status of immunity in chickens when the postvaccination period was in excess of eight to ten days in length.

The procurement of maximal amounts of virus in embryonating eggs was given con-

sideration in the standardization of the vaccines. Factors bearing on the stability of the virus and the circumstances whereby the virus was propagable without apparent alteration, as described in previous sections of this report and in earlier reports, [21, 29] were important, Attention to these factors permitted the almost invariable attainment of titers of D.E.I. virus of at least 10^7 m.l.d.'s /0.05 cc. of the a.a. fluids, minced embryos, or composites of the entire contents (*sans* albumen) of eggs. Since high titers, or 10^{-8}, were usually obtained, titers of 10^{-6} were considered unsatisfactory and related to improper handling of the virus or to other factors.

Other factors that could conceivably have a bearing on the problem of obtaining maximal amounts of virus include the use of seed virus obtained from eggs inoculated with terminal dilutions of the virus, whereby the seed might contain only virus of a variant type. Such virus was described earlier in this report, and the low titers that it attained in eggs were described in a previous report[21] as being unsatisfactory for the preparation of inactivated virus vaccines. The source of the eggs used for production of the virus might also have a bearing on the subject in the preparation of whole egg vaccines, inasmuch as the yolks of eggs laid by FP immune hens have been shown to contain large amounts of specific antibodies.[21] Also it was shown in concurrent studies on Newcastle disease[30] that whole egg vaccines prepared from eggs produced by hens immune to this disease were inferior to those prepared with eggs laid by Newcastle-susceptible hens.

In the absence of information to the contrary, it was deemed necessary to maintain uniformity in the source of the experimental animals used for the assessment of vaccines and in the nutrition and other factors of husbandry. These practices were invoked throughout the course of the investigation.

IMMUNITY RESPONSE TO VACCINATION

Means of Evaluation.—Virus Neutralization by Serum.—The procedure used in conducting this test is described in another report.[21] Briefly, it was conducted by admixture *in vitro* of 0.05-cc. units of serum and of decimal dilutions of virus and by determining the end point of infectivity of the mixtures for 10- to 12-day embryonating eggs. Thus, by comparison with the end point of infectivity of the virus dilutions alone, the number of m.l.d.'s of virus inactivated by 0.05 cc. of serum was established. The D.E.I. virus in the a.a. fluids of lethally infected embryonating eggs was used throughout for this study.

Except in the experiments dealing with the effect of pH and of aging on immunogenicity of the virus, the titers of the serum antibodies were determined in all major studies described in subsequent sections. The tests were made on serums collected uniformly from representative numbers of stock chickens just prior to vaccination and from individuals or groups of chickens that had been vaccinated, or otherwise used in an experiment, just previous to challenge exposure to the virus. In many instances, the information was obtained for successive intervals of from two to four weeks during the postvaccination period.

The antibody titers of the serums of individuals and even of groups of chickens were believed to indicate with considerable accuracy the degree of immunity. However, in comparison to the results obtained by challenge exposure to the disease, the greatest reliability of the test appeared to exist during a four- to six-week period that followed the earliest demonstrability of the antibodies, or at ten to twelve days postvaccination.

Challenge Exposure.—The D.E.I. virus was used to challenge the resistance of the vac-

TABLE 7—Immunogenicity of Variant Virus Administered by Various Routes

Cage No.	Route	Assessment of immunity: Antibody titer, days postexposure 22	42	No. that showed symptoms/ No. exposed intravenously to 10^6 m.l.d. of D.E.I. virus 42 days postvaccination
1	Intramuscular	10^5	10^6	0/5
	Pricking the skin	10^4	10^6	0/5
	Feather follicles	10^4	10^5	0/5
	Controls	0(5)	10^2 or >	?/ 1/6
			10^2	1/1
2	Intranasal	10^4(2, 3)	10^6	0/3
	Proctodaem	10^4	10^6(4)	0/4
	Controls	0(1)	10^5	0/1

*Cannibalism was responsible for death of 6 of the chickens: (1) indicates death at fourteen days; (2), fifteen days; (3), eighteen days; (4), twenty-six days; (5), thirty-six days; and (6), forty-three days.

cinated chickens. The specific source of the virus was serum obtained from adult chickens which had been injected with the virus and were exsanguinated at a late stage of the clinical disease. The virus had been subjected only briefly to propagation in embryonating eggs. Prior to the use of any portion of a preparation, the material was stored at −70 C. in either the lyophilized or fluid form. At the time each portion was used to inoculate chickens, the infectivity titer of the virus was determined for embryonating eggs.

Intravenous injection of the virus was employed in the majority of experiments to challenge the resistance. In one study, the virus was injected intracranially, and, in another, the chickens were placed in close cohabitation with infected chickens and maintained under highly insanitary environment throughout the further course of the experiment. The intravenous route was chosen for most extensive use since in other studies[21] it was shown that this was one of the surest means for establishing the disease in susceptible chickens. Also, this route was considered as most amenable to standardization. Furthermore, the results[21] obtained in susceptible chickens showed that the amount of virus required to cause lethal infection by instillation into the conjunctival sac was about 1,000 times greater than by injection beyond the outer epithelial barriers. At the other extreme, as noted in a subsequent section, exposure by the intracranial route constituted an exceedingly severe challenge and was, therefore, unsuitable as a routine procedure.

The results of the challenge exposures are recorded, for the most part, in terms of death or of survival of the chickens. The occurrence of death within the time when the untreated controls died, along with congestion, hemorrhage, transudation, and other phenomena that typified the acute disease,[20] was considered as evidence of failure to obtain any significant immunity response.

The extension of the survival time of chickens beyond two to three days following parenteral inoculation of the D.E.I. virus was never observed except in cases in which a measure of resistance had been acquired. Evidences of subacute or of chronic forms of the disease were observed in the majority of chickens that had been vaccinated. The subacute forms eventuated in death, usually within ten days postexposure, whereas the more chronic forms showed only a transient dullness and occasional evidence of torticollis.

The lesions that occurred in the subacute or chronic forms have been described[20] previously. The absence or the presence and the degree of macroscopic lesions were considered in all evaluations of the effects of the challenge exposure, while the microscopic lesions and their nature were considered only in the later phases of the work. The gross lesions were present in the majority of chickens that died of the less acute forms of the disease and in many of those that survived or were sacrificed following a two- to three-week period of observation. However, microscopic lesions that were termed immune reactions were observed in the majority of the birds that showed neither symptoms nor gross lesions. Since the relatively avirulent variants of the D.E.I. virus often produced both gross and microscopic lesions of a comparable nature when injected alone, the value of the pathologic criteria was minimized when the variant vaccinates were challenged with the D.E.I. virus within several weeks. These studies, particularly of the histopathology, were of suggestive value in making finer distinctions between the immunizing capacities of various vaccines and indicated the occurrence of certain host reactions in the degree of immunity obtained by the procedures employed. For all practical

TABLE 8—Retention of Immunogenicity by Formalinized Nonadjuvant Vaccines of Different pH Values During Storage at 6 C. for Intervals up to 611 Days

Vaccine		No. of chickens survived/No. exposed intravenously to 10^4 m.l.d. of virus two weeks after the intramuscular injection of 1 cc. of vaccine of the following ages, in days												Totals	
No.	pH	16	37	59	79	113	142	175	211	257	365	471*	611	No.	(%)
61	5	0/2	0/2**											0/4	0
	6	2/2	2/2	3/3	3/3	3/3	3/3	1/3	2/3	0/2	3/3	5/5	3/5	3/5	33/42	79
	7	2/2	2/2				3/3	3/3	1/3	1/2	3/3	3/5	2/5	4/5	24/33	73
	8	2/2	2/2	3/3	3/3	3/3	4/4	3/3	3/3	1/2	2/3	2/4	3/5	3/5	35/43	82
	9	2/2	2/2	3/3	3/3	3/3	3/3	3/3	2/2	2/2	3/3	3/5	2/5	3/4	34/40	85
TOTALS:															
(No.)		8/10	8/10	9/9	9/9	9/9	13/13	10/12	8/11	4/8	11/12	14/20	10/20	13/19	126/158	80
(%)		80	80	100	100	100	100	83	73	50	92	70	50	68		

*The first group was challenged fourteen days postvaccination, the second group forty-nine days postvaccination.

**An additional 5 vaccinated chickens also died of the disease following exposure to smaller amounts of virus.

purposes, however, failure of the challenge exposure to interfere visibly with the normal functions was taken as evidence of a substantial and commendable degree of immunity.

Influence of Age of Chickens.—No factor associated *per se* with maturation has been suggested to be important in the native susceptibility of chickens to fowl plague or in the acquisition of resistance to it. However, it has been observed[27] that chicks hatched from eggs laid by hyperimmunized hens had a yolk-transferred passive immunity for about four weeks and that this interfered with successful active immunization. The presence of the antibodies in FP-immune hens was confirmed in our study.[21] In concurrent studies on Newcastle disease virus,[36] the presence of specific antibodies in the yolk and their importance during the early weeks established the occurrence of a condition that was analogous to that observed earlier for fowl plague.

Our studies were directed primarily at an evaluation of the effect that age itself might have on the immunity response. This was of immediate importance in expediting the studies because of a shortage of facilities for maintaining suitable numbers of experimental chickens.

Evidence obtained early in the investigation indicated that the initial response to vaccination in chicks up to 2 months of age was essentially the same as in mature chickens. The refractivity of the vaccinated chickens to the challenge exposure in one of the earlier experiments, along with that of a subsequent and confirmatory trial, is shown by the data in table 3. While not recorded in this table, the titers of serum antibodies bore out the close relationship of the responses of chickens of the various age groups to the vaccination procedures employed. The titers of the group-composite serums in the first experiment were 10^2 in 4 cases, 10 in 1, and 0 in 2, and in the second test these were 10^4 or 10^5 in ten groups, and 10^3 and 10^2 in the two other groups. The higher titers were not correlated with any age factor.

TABLE 9—Immunogenicity of FP Vaccine No. 61 Following Storage for 471 Days, as Determined by Prechallenge Antibody Titers and by Postchallenge Lesions

pH of vaccine	Challenged 14 days postvaccination				Challenged 49 days postvaccination			
		14-day survivors				14-day survivors		
			Positive				Positive	
	Antibody titers	No.	Gross	Histologic	Antibody titers	No.	Gross	Histologic
6	10^4	5	3	2	10^2	3	2	3
7	10^3	3	1	3	10^2	2	1	0
8	10^2	2	2	2	10^3	3	2	3
9	10^3	3	0	3	10^2	3	2	3

TABLE 10—Immunogenicity of Several FP Formalinized Vaccines Administered Intramuscularly in 1-cc. Amounts

Embryo material	Challenge exposure		No. remained healthy/No. exposed
	Days postvaccination	m.l.d.	
a.a. fluid	40	10 to 10^3	7/10
	60		4/7
	80		8/8
	120		7/9
	150		3/10
a.a. fluid + minced embryos	17	10 to 10^5	22/25
	42		19/26
	70		16/25
	98		16/25
a.a. fluid + minced embryos	21	Simulated natural means	8/10
	60		9/9
	90		3/9
	120		3/6

The data indicated also that the response to vaccination was comparable in chickens of a light and of a heavy breed and in both sexes. The hens in the first test were White Rocks, whereas those in the second test were White Leghorns. In the 1-week-old groups in both tests and the 1-month-old group in the second test, both sexes were about equally represented; all other chickens used in the study were males.

Influence of Dosage.—Numerous observations were made on the comparative immunogenicities of different amounts of virus units or m.l.d.'s of the inactivated and the living D.E.I. and variant viruses. The different forms of the viruses were injected either alone, with the exception of the living D.E.I. virus, or were followed by a subsequent injection of the same or of another virus form. The inactivated D.E.I. virus was employed both with and without the addition of adjuvants.

Single Doses of Inactivated Virus.—The data recorded in table 4 indicated that 20 x 10^8 units of the formalinized D.E.I. virus in a.a. fluid was the approximate minimal amount that would engender in chickens significant levels of serum antibodies and provide some protection against a challenge exposure ninety-eight days later. The data shown for one of the variant strains are suggestive of an equality with the parent virus on the basis of antibody titers but of a superiority in providing refractivity to the challenge exposure. This apparently greater response to 20 x 10^7 units of the variant might, however,

have been related to a retarding or adjuvant effect of the tenfold larger volumes of vaccine that were injected in equalizing the virus unit dosage with that of the D.E.I. strain. In other experiments on non-adjuvant vaccines, evidence was obtained to the effect that at least 20 x 10^7 units of virus in a.a. fluid were necessary to provide refractivity to challenge exposure fourteen to twenty-one days later.[20] Also, one decimal smaller amount of virus in embryo pulp apparently accomplished the same end.[28]

The production of substantial degrees of immunity with as low as 5 x 10^5 units of virus was demonstrated following incorporation of the virus in certain oily adjuvants. This is shown in the data on vaccines 96, 97, and 98 in table 13, along with evidences of increased immunity responses to the vaccines composed of two decimal increments of virus material. While antibody responses were not demonstrated for any of the lowest dosages of the three vaccines within twenty-one days postvaccination, two of them provided considerable resistance against the challenge exposure. These findings agreed essentially with those of other experiments that are described in later sections.

Single and Divided or Doubled Doses of Inactivated Virus.—In subsequent sections dealing primarily with adjuvants and duration of immunity, information is recorded that has a bearing here. The immunity responses to 12 x 10^6 units of formalinized virus when injected in a single dose or in divided doses separated by an interval of four weeks are summarized in table 10 and figure 2. A comparison of the refractivity to challenge exposure after one hundred days for vaccines 89 and 90, given in single and divided doses, respectively, are recorded in the table. While the former procedure protected 2 of 5 chickens and the latter 3 of 4 chickens, the results recorded graphically in the figure for the antibody titers at five periods during the study revealed marked differences between the two procedures. The overall antibody response appeared, likewise, to favor the procedure of divided doses.

The information obtained for several groups of chickens in another study, and recorded in table 15, involved a comparison of the effect of single doses and of a double, or repeated dose (after 21 days), of an adjuvant vaccine. The data obtained on these groups, namely 1, 2, and 10, showed rather uniformly high immunity responses. No significant differences were detected by the procedures employed even though the prechallenge period was of nearly five months' duration.

Living Virus.—This study consisted of an evaluation of the immune responses to various amounts of the living D.E.I. virus and of the living variants.

TABLE 11—Immunogenicity of Formalinized Infected Embryo Components with and without the Addition of Adjuvants

Experiment No.	Vaccine*				Response**	
	No.	Composition	Method of preparation	Dosage cc., i.m.	Antibody titers	No. remained healthy/No. challenged
1	72	F	Dilution	1	0-10	0/3
	69	F + A	Manually in mortar	1	10^4-10^5	3/4
	70	F + AM			10^4-10^5	3/4
	71	F + A^3M			10 -10^6	2/3
	68	F + A^3			1 -10^2	1/4
	66	FP	Dilution	1	10 -10^2	2/4
2	88	F^a	Dilution	1	10	2/5
	89	P^a		1	10^2	2/5
	90	P^b		2 x 1	10^2	3/4
	77A	WE^b		1	10	2/5
	83	F^a + A	Manually in mortar	1	10^4	3/4
	82	F^b + A^4		2	10^4	5/5
	85	P^a + A		1	10^5	4/6
	84	P^b + A		2	10^4	2/5
	91	WE^a + A		1	10^4	3/3
	87	F^b + A^4	Light shaking	2	10^4	6/6
	86	F^b + A^4	Mixer	2	10^4	6/6

F = a.a. fluid. P = Embryo pulp. WE = Whole egg content *sans* albumen. A = Adjuvant (Falba and mineral oil). A^2 = Adjuvant (Falba and peanut oil). A^3 = Normal embryo mince (20%). M = *Mycobacterium butyricum*, 140 mg./100 cc.

*Under composition, the following percentage of volumes pertain to the two experiments: Experiment 1: 50 per cent virus material (Vaccine 68 = 30%F, 20%P), 10 per cent Falba, 40 per cent oil, and other volumes obtained with saline; Experiment 2: virus material either [a]60 per cent or [b]30 per cent; adjuvants Falba 10 per cent and mineral oil either 30 per cent or, in A^4, 60 per cent; and other volumes were obtained with saline.

**The responses were determined forty-two and one hundred days postvaccination in experiments 1 and 2, respectively; the challenge exposure in the first experiment was 10^3 m.l.d. and in the other 10^2 m.l.d. injected intravenously. Nine of 10 controls died that were injected with decimal end point dilutions of the larger inoculums.

The data in table 5 show the augmentation in antibody titers and in refractivity to severe intracranial exposure to the D.E.I. virus which this virus provoked following its intravenous injection into relatively resistant chickens. Of 31 chickens that had previously withstood intravenous exposure, 29 resisted the intracranial challenge. Of the 20 chickens that had received the inactivated virus alone intramuscularly, none of the group of 4 that were injected with the severest intracranial challenge survived. Of the 12 others injected with smaller inoculums, 1 resisted. The decimal increases from 10 to 10^5 m.l.d.'s of virulent virus used in the intravenous exposures were reflected neither in the antibody titers nor in the resistance to intracranial exposure.

In later studies, the antibody titers that ensued when intravenous injection of the D.E.I. virus was superimposed on the higher initial response to an adjuvant vaccine were not noticeably at variance with those produced by the inactivated virus alone (*see* table 15).

The influence of dosage of the living variants on the resultant immunity was determined in fully susceptible chickens and in those previously injected with inactivated virus. The results of several tests summarized in table 6 seemed to warrant the following deductions: (1) dosages of the 4395 variant from 20 x 10^5 to 2 embryo m.l.d.'s, by decimal decrements, were equally immunogenic within a 21-day test period; (2) similarly varied amounts, from 10^7 to 0.1, of the 19493 variant resulted in immune responses that were not uniform but roughly proportionate to the size of the inoculums within a 105-day postvaccination period; and (3) the results obtained with the latter variant were of a lower order than those of the 21-day test or of a subsequent 105-day test with one dose of the 4395 variant. The superiority of the 4395 over the 19493 variant as a vaccine was also borne out by histologic examination. Lung, liver, spleen, and kidney tissues were obtained twenty-one days following the challenge exposure from the 9 chickens that received the 4395 variant in the 105-day test and from the 6 that were initially injected with 10^7 and 10^8 m.l.d.'s of the 19493 strain. Lesions were found in 2 of the former group and in 5 of the latter group. The distribution, as well as the intensity, of partial fowl plague immune-challenge reaction was much greater in the 19493 vaccinates than in the others.

Further data on the immunogenicity of the 4395 variant are recorded in a subsequent section and in table 15.

Influence of Route of Administration.—In studies by Hallauer,[9] the injection of formalinized chicken tissue vaccines by the i. v. route appeared to provide an immunity that was superior to the injection of the same vaccines by the intradermal (i.d.) route. By further comparison of the resistance of chickens to challenge exposure after fourteen days, the responses to intramuscular (i.m.) vaccination and to the injection of incomparably smaller doses intracerebrally were less beneficial than with the i.v. procedure. The i.v. route was also found superior to the i.m., and, in addition, superior to subcutaneous injection, by Nakamura, *et al.*[10]

Two direct comparisons of the influence of vaccination route on the immunity response to inactivated D.E.I. virus were conducted. The results indicated that the refractivity to challenge exposure was essentially the same twenty-one days following i.d. and i.m. vaccination,[28] but that the rate of development of immunity was apparently slower following vaccination by the i. d. route than by the i.m. (*see* table 14).

The intramuscular route was employed almost exclusively in studies of the inactivated vaccines. This was prompted by (1) limita-

TABLE 12—Comparative Adjuvant Effect of Alumina Gel and of Falba and Mineral Oil on Formalinized Plague Virus Vaccines 21 Days Following Intramuscular Injection of 1 cc.

Antigen content* (%)	Adjuvants		Antibody titers	No. remained healthy/ No. challenged i.v. with 10^5 m.l.d. of virus	No. with gross lesions 2 weeks postchallenge/ No. remained healthy
	Name	Amt. (%)			
70.0	Alumina gel.	30	10^8	10/10	8/10
7.0			10^6	9/10	5/9
0.7			10^5	7/9	5/7
70.0		15	10^4	8/9	4/8
7.0			10^5	8/10	1/8
0.7			10^5	8/10	4/8
70.0	Falba and mineral oil	5	10^2	7/7	3/7
7.0			10^4	7/8	2/7
0.7		25	10^4	5/8	5/5
70.0	None		10^3	6/9	4/6
Nonvaccinated controls			0	0/3	

*Where necessary, an 0.85 per cent sodium chloride solution was added to the egg components to fulfill requirements of volume.

tions imposed by the immediate or subsequent effect of the injection of the egg components themselves or of the products obtained after admixture with adjuvants, as mentioned in a previous section, and (2) by considerations of practical means for injection.

An evaluation was made of the route factor on the immunizing propensities of the living 4395 variant. The summary of the results in table 7 appeared to justify an interpretation that high and equal immune responses followed the injection of the virus by the i.m. and intranasal (i.n.) routes and its application onto feather follicles, onto the surfaces of the proctodaeum, and into the skin and subjacent tissues. The transmission of the virus to the controls, which appeared to be related to the factor of cannibalism, was believed not to have altered significantly the immune responses to actual exposure of the virus. Of further interest, in view of the uniformity of immune responses, were the necessarily unequal doses of the virus that were administered. The inoculum containing 20 x 10^6 embryo m.l.d.'s of virus per cubic centimeter was given in 0.1-cc. amounts by the i.m. and i.n. routes, whereas a needle and a swab were dipped into the fluid for application, respectively, by pricking the skin and to the feather follicles and the bursal surfaces.

Influence of pH and of Embryo Components.—The association of loss of immunogenicity with the disappearance of viability due to suboptimal conditions of pH was observed by Eaton[39] for an influenza virus. This finding, together with the establishment by Pyl[40] that the optimal range for the viability of the FP virus existed between approximately pH 6.3 and 9.1, which found general confirmation in our work,[39] suggested the desirability of associating different pH levels with studies on the keeping qualities of vaccines.

Vaccines of different unit values of pH from 5 to 9, inclusive, were studied. The final composition of the vaccines included formalinized a.a. fluids and minced embryos in 40 and 20 per cent concentrations, respectively, and 40 per cent of buffer[41] adjusted and added in a manner to provide the desired pH levels of exposure. Each cubic centimeter of vaccine contained 20 x 10^6 units of virus. Portions of the vaccines were stored at 37 C. and at 6 C. The comparative immunizing properties of the vaccines were tested at intervals by injecting 1 cc. i.m. and challenging the resistance two weeks later by i.v. exposure to 10^4 m.l.d.'s of virus. With the exception of the pH 5 vaccine which was discarded after the second test due to loss of activity, all others stored at 37 C. were subjected to nine tests, covering a period of 257 days, or until the materials were depleted. The results of the nine tests were comparable to those of similar tests of the vaccines stored at 6 C. Three additional routine tests of the vaccines stored at 6 C. were conducted, making a total of 12 tests in all, covering storage for 611 days, for which the results are given in table 8. The data appeared to warrant the major deductions that at pH 5 the value of the vaccine was lost within sixteen days while a large measure of the im-

TABLE 13—Immunogenicity of Virus Inactivated by Formalin and by Ultraviolet Light of Two Types (Exposure to Wave Lengths of 2,537 Å and of 1,600-1800 Å for Periods of 20 Minutes and from 1.1 to 3.8 Seconds, Respectively)

Vaccination intramuscularly		Assessment of immunity 14 to 21 days postvaccination		
Vaccine	Original virus material in vaccine, cc.*	I.v. challenge with virus, m.l.d.	Antibody titer	No. remained healthy/No. challenged
65A u.v. 20 min.	1.0	10^4	not determined	0/5**
	0.1			0/4**
65B formalin	1.0			4/6
	0.1			2/6
92 u.v. 1.1 sec.	0.5	10^2-10^6	10^4	12/12
	0.1		10^3	11/12
93 formalin	0.5		10^4	12/12
	0.1		10^3	12/12
96 u.v. 3.0 sec.	0.5	10^5	10^2	3/3
	0.05		10	5/6
	0.005		0	5/6
97 u.v. 3.8 sec.	0.5		10^2	3/3
	0.05		10	6/6
	0.005		0	1/6
98 formalin	0.5		10^4	3/3
	0.05		10^2	6/6
	0.005		0	4/6

*Vaccines 65A and B were nonadjuvant vaccines, whereas all others were admixed, after necessary additions of saline solution, with an equal part of a combination of Falba and the mineral oil which gave final concentrations of 7.5 per cent and 42.5 per cent, respectively, of the adjuvants.

**Three chickens of the original 6 in these groups died from active virus that was still present in the vaccine.

munogenicity was maintained at the other pH levels for the entire test period. Irregularities suggestive of deterioration were not encountered until after 142 days of storage. The final pH values of the vaccines tested extensively were 6.1, 7.1, 7.65, and 8.6. After the cine prepared by substituting an 0.85 per cent same interval, a pH of 7 was found in a vaccine prepared by substituting an 0.85 per cent sodium chloride solution for the buffer.

On the eleventh test, or after storage for 471 days, of the vaccines of different pH values, supplementary assessments were made of the immunity responses. The antibody titers were determined for the groups submitted to challenge exposure at the usual time and prior to the challenge of an additional group five weeks later. The results of these tests along with observations on the postchallenge residual pathology are recorded in table 9. Summary considerations of survival, antibody titers, gross lesions in the liver and spleen, and microscopic lesions in lung, liver, spleen, and kidneys indicate that vaccines of pH 6 and 7 were somewhat superior.

Embryo Components. — Data presented in other sections are pertinent to this subject. It was related earlier that suitable concentrations of virus, of 20 x 10^7 m.l.d.'s or more, were obtainable in all components of embryonating eggs with the exception of the yolk, but that inclusion of the yolk with the other components did not reduce the amounts of titrable virus to objectionable levels. Also, reference was made in considerations of graded doses of vaccines to the equal or possibly greater immunogenicity of inactivated virus in minced embryos as compared to that in a.a. fluid, even though the demonstrable amounts of infective virus were usually one decimal dilution lower in the former. The possible influence of yolk-borne antibodies has also been mentioned. Further information bearing on this subject is given in the next section.

Influence of Adjuvants. — As previously mentioned, the need for enhancing the immunogenicity of the inactivated virus and the prospects for doing so, prompted the trial of various materials for possible adjuvant effects.

Data obtained in several studies on nonadjuvant vaccines and with postvaccination periods in excess of two months are given in table 10. With postvaccination periods less than approximately sixty days, 69 of 87 chickens (80%) resisted the challenge exposure. However, with prechallenge periods up to 150 days, 56 of 82 chickens (61%) were refractive to the challenge exposures. Both the refractivity to challenge exposure in the earlier periods and the evidences of its later diminution emphasized the need for enhancement of the immune responses.

The results of the first test of the adjuvant vaccines (*see* experiment 1, table 11) demonstrated the superiority of these vaccines over those without adjuvants. These results would appear to justify the deductions that (1) the incorporation of Falba and mineral oil into the vaccine greatly enhanced the immune response; (2) neither the substitution of peanut oil for mineral oil nor the addition of killed, acidfast bacteria showed any significant advantage; and (3) that the addition of normal embryo pulp to the virus material was devoid of any significant adjuvant effect. These vaccines contained 2 x 10^9 units of virus.

The extension of the study was designed to determine the possible effect of the method of

TABLE 14—Time Required for the Development of Immunity to FP Following Vaccination by Different Vaccines and Methods

Vaccination procedure			Assessment of immunity*					
Vaccine			Challenge exposure to D.E.I. virus	S/E ratio, days postvaccination				
Type	Amount	Route		Not vaccinated	0	2	4	6	8-12
Formalinized a.a. fluid	1 cc. (2 x 10^9 U.)	i.m.	10^4 m.l.d., c.s.				2/2	2/2	6/6
		i.d.					0/2	1/2	6/6
Formalinized adjuvant vaccine**	1 cc. (2 x 10^9 U.)	i.m.	1-10^4 m.l.d., i.v.	1/6	0/6	0/5	2/6	4/6	18/18
			Simulated natural exposure	2/3	1/3	0/3	3/3	1/3	9/9
Living variant 1395	10^2 embryo m.l.d.	i.v.	1-10^4 m.l.d., i.v.	0/6		3/6	6/6	6/6	17/17
			10-10^3 m.l.d., i.v.	0/4		6/18	17/18		

S/E = Ratio of number survived to the number exposed. i.m. = intramuscular. c.s. = conjunctival sac. i.d. = intradermal. i.v. = intravenous. U = units.

*Neutralizing antibodies were first demonstrated in serum composites from 6 chickens, as follows: 10^1 titer for the adjuvant vaccine at 12 days postvaccination; 10^2 titer for the living variant at 10 days postvaccination.

**The vaccine was comparable to No. 70, described in table 11, except that the virus material consisted of 30 per cent a.a. fluid and 20 per cent minced embryos.

addition of Falba and mineral oil as well as to test other formalinized components of infected eggs. The procedures involved in the preparation of the vaccines and the results obtained are described in a previous section and are indicated in table 11, experiment 2. The vaccines contained either 6 x 10^8 or 12 x 10^8 virus units, and the amount of virus each chicken received was equalized by injection of a single 1-cc. or a single 2-cc. dose, or, in the case of vaccine 90, two 1-cc. doses with an intervening period of four weeks. Again, the results showed greater responses to the adjuvant vaccines. Their superiority here appeared to be unrelated to differences in the methods used in incorporating the adjuvants and also to the source of the virus material, that is a.a. fluid, minced embryos, or whole egg content. The immunity produced by the divided doses of the nonadjuvant vaccine (number 90) was greater than that obtained with a single injection of an equal amount of the same virus material (number 89). This is further evidenced by the results of periodic antibody tests as shown in figure 2, as well as the intermediate position of the response between that of the vaccines with and without adjuvants.

The observation, in the preceding experiment, that the three vaccines (numbers 82, 86, and 87) containing 0 per cent mineral oil produced a consistently high resistance to challenge exposure was subjected to further inquiry. The study was pursued satisfactorily only to the stage of determining antibody titers at thirty to sixty days postvaccination. These suggested that a vaccine containing only mineral oil (30%) as adjuvant was equal immunogenically to a vaccine containing both mineral oil (22.5%) and Falba (7.5%). The antibody responses to both vaccines were typically higher than those produced by the nonadjuvant vaccine.[42]

The comparative responses to formalinized virus in whole egg content (20 x 10^8 units per cubic centimeter) admixed with alumina gel and with Falba and mineral oil are demonstrated in table 12. Within the twenty-one-day postvaccination period, any significant differences that existed in antibody response and in refractivity to challenge exposure appeared to be somewhat in favor of the alumina gel vaccines. Also, the response to the vaccine without adjuvants but with maximal content of virus appeared to surpass only that of the one containing Falba, mineral oil, and a virus content smaller by two decimal decrements.

The enhancement of immunogenicity by Falba and mineral oil is demonstrated further in subsequent sections.

Influence of Method of Inactivation.—The procedures and the results of irradiation of the D.E.I. virus by u.v. light of two types have been described in a previous section. Other portions of the same virus materials as those subjected to the treatment were inactivated with formalin. Comparative immunogenicity tests were conducted on the materials. The experiments are summarized in table 13.

As is there shown, virus inactivated by the longer rays of the Hanovia coil displayed little, if any, immunizing capacity. While all of the 9 chickens died following challenge exposure to the virus fourteen days postvaccination, the course of the disease, not shown in the table, suggested that some resistance had developed. Thus, the chickens injected with the larger amount of the u.v. and the formalin-treated material and that died following exposure, survived three and eight-tenths and seven and five-tenths days, respectively; 2 nonvaccinated controls died twenty-four hours postchallenge. The fact that the u.v. material itself caused the disease in 3 of the 12 chickens indicated a close association of the lability of infectivity and immunogenicity to the light.

In clear contrast, the virus inactivated with the apparatus of Oppenheimer, Levinson, and associates[34] possessed an antigenicity that compared favorably to that treated with formalin. A direct comparison of material exposed eight-tenths of a second longer than another portion (vaccines 96 and 97) demonstrated probable evidence of a deleterious effect on the virus. However, within the limits of the experimental procedure, the use of the u.v. light failed to display any advantage over the convenient and more easily controlled use of formalin.

Influence of Stilbesterol Treatment of Cockerels.—A desire to quiet the crowing of cockerels was responsible for a study of the influence of treatment with diethystilbesterol on the response to vaccination. Pellets of the synthetic hormone* were placed beneath the skin of 54-day-old cockerels. Treatment with one 15-mg. pellet per chicken at the time of vaccination with formalinized adjuvant vaccine was without demonstrable effect on the immune response that developed within forty-two days, when compared to that of cockerels without the treatment. Likewise, the hormone did not alter the course of the disease in nonvaccinated or control chickens.[43]

Time for Development of Immunity.—Several experiments were conducted (*see* table 14) to ascertain the length of time required for the appearance of immune responses following vaccination. Challenge exposure of all of the chickens of a given experiment was made at the same time or following vaccination of all groups and the passage of the stated intervals. Group composites of blood were collected just prior to the exposure to

*Obtained from Lederle Laboratories, Inc.

the D.E.I. virus. While significant evidence of refractivity to the disease was demonstrated as early as two days postvaccination, an additional period of six days was required before uniformity was obtained with all of the procedures. Serum antibodies appeared in significant titers (neutralizing capacity for 10 or more m.l.d.'s of virus/0.05 cc. of serum) at ten to twelve days postvaccination.

The degrees of the demonstrable immune responses were related to the vaccination procedures and, in the case of resistance to challenge exposure, to the size of the challenge doses of the D.E.I. virus. Thus, the results indicated that the response to the inactivated virus was slower to develop following vaccination by the i.d. than by the i.m. route. Refractivity to challenge exposure was demonstrable four days after administration of the adjuvant vaccine by the i.m. route. The most rapid response was obtained by the i.v. injection of the living variant virus; resistance to the exposure to D.E.I. virus was evident at two days postvaccination. Comparable degrees of resistance were present four days postinjection of the living variant and eight days after injection with the adjuvant killed virus vaccine. The presence of significant antibody titers in the serum occurred first (between eight and ten days) in response to the injection of the variant and preceded the appearance of similar responses to the inactivated virus by at least two days.

The presence of degrees of immunity during the developmental stages was reflected by the resistance shown to the various challenge exposures to the D.E.I. virus. This was suggested in the test with the adjuvant vaccine and graded doses of challenge virus in that 2 chickens that died in the group vaccinated six days previously had received the maximal challenge in one case and the intermediate challenge (10^2 m.l.d.'s) in the other. At the four-day period, the 2 chickens that survived had received the larger and the smaller inoculum, respectively. The differences between these results and those obtained by simulated natural exposure were attributed to irregularities associated with the latter means of exposure. The effect of graded doses of challenge virus was shown best in connection with the immunity responses to the injection of the living variant. In the two-day test, the 2 chickens challenged by the smallest dose and 1 of the 2 by an intermediate dose of 10^2 m.l.d.'s survived. The second experiment of this test eventuated in the survival of the 6 chickens challenged by the smaller dose of 10 m.l.d.'s while death occurred in all chickens of the groups which had received 10^3 or 10^5 m.l.d.'s of the D.E.I. virus. Of the chickens that failed to withstand the challenge exposures, the course of the disease was not significantly prolonged as compared to the nonvaccinated or control animals.

Duration of Immunity.—Certain studies reported in previous sections have a bearing on this subject. The injection of living D.E.I. virus following vaccination with inactivated virus provided a high degree of resistance to intracranial exposure to the virus, and, in addition, showed that by the test procedure used

TABLE 15—Immunity Response to Various Vaccination Procedures during a Period of 147 Days

Group No.	Vaccination procedure: Intramuscular vaccination, day of experiment 1	Vaccination procedure: Intramuscular vaccination, day of experiment 21		Assessment of immunity: Log of group antibody titers, days of experiment 21	42	63	105	147	No. survived/No. challenged, 147th day of experiment, D.E.I. virus, i.v., 10^5 m.l.d.
1	Vaccine 115, 1 cc. (70% formalinized whole egg, 25% min. oil, 5% Falba, 14 x 10^8 virus units)	None		5	5	6	5	5	5/5
2		Vaccine 115, 1 cc.		5	6	6	6	6	5/5
3		D.E.I. virus m.l.d.	10	4	5	5	5	5	6/6
4			10^3	4	6	5	5	5	4/5
5		Variant 4395 embryo m.l.d.	1	5	6	6	4	6	4/6
6			10^2	4	6	5	5	5	5/5
7			10^4	4	6	6	5	5	5/5
8	None		1	0	5	5	6	6	4/4
9			10^4	0	5	5	6	7	6/6
10		Vaccine 115, 1 cc.		0	5	5	4	4	5/5
11* A		None		0	0	0	0	0	0/3
11* B				0	0	0	0	0	0/3
12				0	0	0	0	0	0/6

*The chickens of group 11A were caged with those of groups 3 and 4; those of 11B were caged with groups 5, 6 and 7; groups 1, 2, and 10 were maintained in isolation in an environment where evidences of the FP disease were never encountered.

the maximal antibody concentrations attainable may be represented by titers of 10^6 to 10^8 (table 5). The living variants, particularly the 4395 strain, were shown to engender high titers of antibodies and to provide noteworthy resistance to challenge exposure to the D.E.I. virus 105 days later (table 6). The study summarized in table 10 was designed to determine the durability of the immunity that followed vaccination with the nonadjuvant vaccines. Irregular immunity response, demonstrable during the two months following vaccination, and appreciable decreases in resistance after two to three months prompted the direction of attention to the adjuvant vaccines. The superiority of the vaccines containing the oily adjuvants over those of the simpler type was shown in early experiments and for a postvaccination period of one hundred days (table 11).

With the preceding information available, evaluation was undertaken of procedures selected for the high quality of immune responses or for their possible merits in practical application. A vaccine was prepared from formalinized, infected whole egg contents, mineral oil, and Falba. The immunity that a single dose of this vaccine could provide was studied in comparison to that resulting from (1) a second dose of the same vaccine, (2) a second injection with either living D.E.I. or variant virus, and (3) an injection of the variant virus alone. The study was extended over a postvaccination period of 147 days and is summarized in table 15.

The results recorded in the table showed that comparably high antibody titers developed and were maintained in response to each of the 10 procedures employed, and likewise that a high degree of refractivity was present to severe challenge exposure at the end of the study.

Accordingly, no apparent benefit was revealed from the supplemental injection of the inactivated virus vaccine, the living D.E.I. virus, or the living variant virus. Further, significant superiority of the use of living variant virus alone in graded amounts over that of the inactivated D.E.I. virus was not demonstrated. The 3 deaths in the total of 52 vaccinated chickens occurred in groups that received the supplemental injection of a large amount of the living D.E.I. virus (1 case) and of a small amount of the variant virus (2 cases). The 49 chickens that remained healthy were sacrificed sixteen days after the challenge exposure and examined for residual macroscopic evidences of infection by the virus. Lesions were present in 32 (65%)."

DISCUSSION

The demonstration, in the initial stages of this laboratory investigation, that formalinized components of embryonating chicken eggs lethally infected with the fowl plague virus increased significantly the immunity of chickens against infection by the virus, prompted immediate extension of the work along this line. Attention was given concurrently to factors having a bearing on the maintenance of suitable virus, to means for more critical assessment of the resultant immunity, and to matters of possible importance in practical application.

The procedures involved in the storage and propagation of the virus, as described previously[21] or herein, were designed to guard against, or insure the recognition of, adverse alterations in the nature of the virus. However, the counterpart of this circumstance, or the possibility that the classical, highly lethal virus might be sufficiently altered for use as a vaccine in the living state, was considered as an interesting prospect. Assessments on the stability of the D.E.I. virus were scarcely begun when a variant strain of virus was isolated. The relative avirulence of this variant for chickens and its high immunogenicity provided the opportunity for extended study of the immune response to it in comparison with that to the inactivated parent virus. Another variant strain was also given consideration. Alterations in the D.E.I. virus or in one of the variants were not detected in studies conducted to challenge their stability.

The procurability of large amounts of virus was of concern mainly with the D.E.I. virus, which served for the preparation of inactivated virus vaccines. While the virus was capable of producing lethal infection in embryonating eggs following its introduction by various routes, a critical study of the concentrations attained in the various components of the egg was made for only two of the routes. A somewhat more rapid production of virus and shorter course of disease followed injection of the virus intravenously, but the relative simplicity and final virus concentration attained by the other procedure favored its selection for use in vaccine production.

The apparent greater immunogenicity of minced embryos as compared with extra-

embryonic fluids, on a virus unit basis, may be explainable by limitations in demonstrating intracellular virus and by a longer retention of the injected antigen. The virus concentrations of both of these components and of other membranes were within the standards suggested for vaccine production. As predictable from a simple 1:2 dilution, the addition of the yolk to the other components in later studies did not perceptibly alter the titer of virus demonstrable prior to inactivation or the effectiveness of the finished vaccine.

The low concentrations of the variant strains that developed in embryonating eggs suggested their unsuitability for use as killed virus vaccines. This was confirmed by observations on the immune responses of chickens to one of the variants inactivated by formalin.

The study of the major types of immunizing procedures was carried out insofar as the circumstances of the project permitted. On the important phases of the rapidity of development, degree, and persistence of immunity, the following deductions appeared tenable.

1) The response was most rapid following injection of living variant virus and was somewhat slower to develop following administration of the inactivated virus. Incorporation of the virus in certain absorbent materials appeared to retard the response still further. The possibility of association of the rapid response to the living variant with a blockade effect, considered to exist between other living as well as inactivated viruses,[45] is regarded as likely. However, an interference effect of the living variant on the course of the infection by the D.E.I. virus in embryonating eggs was not demonstrable, although there was suggestive evidence that ultraviolet-inactivated, but not formalinized, virus may provide such interference.[42]

2) In the order of least to greatest influence on all features of the immune response, the procedures employed may be arranged as follows: (a) inactivated nonadjuvant vaccines, either in single or divided doses; (b) inactivated adjuvant vaccines; (c) living variant virus; and (d) living D.E.I. virus following an initial response to inactivated virus. This deduction is substantiated by comparisons of serum antibody titers, which reflected the general degree of immunity quite accurately, and by the results of challenge exposures to the virulent virus. While it was expected that group or average antibody titers might not indicate the specific refractivity to challenge exposure of chickens within the group, instances were observed in which the results of serum tests of individual birds did not fairly reflect their refractivity. This was especially evident with postvaccination periods beyond four to six weeks. The possible presence of other factors inherent in the nature of the host was suggested by irregularities in the quality of immune responses within individual experiments.

Inconsistencies in refractivity to challenge exposures in several experiments were such as to suggest strongly an inverse relationship to the size of the challenge dose of virus. The details of the studies, along with an experiment designed to test the possible significance of the observation were presented in Interim Report No. 7.[46] The results indicated that the greater mortality to the smaller challenge doses in the earlier experiments was due to chance variations in the immune responses of the individual chickens.

Irregularities in the response to the virus were observed that seem particularly to merit further study. In this regard, the apparent suppression of the antibody titer reaction to the D.E.I. virus by its injection during the early, pronounced response to adjuvant vaccines was of interest. Also, an apparently diminished refractivity to challenge exposure in a few individuals, occasioned by attempts to fortify the basic immunity to adjuvant vaccines by exposures to living D.E.I. or variant virus during the initial ascending response, was without obvious explanation.

It should be emphasized that the challenge exposures of the vaccinated chickens were undoubtedly much more severe than those likely to be encountered under natural conditions. Under the conditions of the studies, evidence that the chickens were less

than solidly immune, even with high antibody responses and in connection with prior exposure to the living virulent virus, were shown by the gross and, particularly, the microscopic postchallenge pathology. However, this is considered to be no serious objection to the belief that the single injections of the adjuvant vaccines or, in an enzoötic area, of properly selected variant strains would be highly useful procedures for combating the disease in the field.

SUMMARY

A description is presented of a laboratory investigation on the immunization of chickens against fowl plague. A Dutch East Indies strain of virus and two variant viruses isolated from it were employed. Major considerations were given to defining optimal circumstances for the preparation, preservation, and use of vaccines, as well as to determining the attributes of the vaccines. The data support the following conclusions:

1) The high concentrations (usually 20 x 10^8 m.l.d.'s per cubic centimeter) attained by the D.E.I. virus in most components of embryonating chicken eggs were suitable for the preparation of inactivated virus vaccines. Its high virulence for chickens prevented a definition of its immunizing capacities by injection alone into fully susceptible chickens.

2) The titers (commonly 20 x 10^6 by intravenous test in embryos) attained by the variant viruses in embryonating eggs were unsatisfactorily low for immunogenic purposes following inactivation, but the relative avirulence of the variants for fully susceptible chickens permitted studies on their immunizing capacities.

3) The infectivity of the D.E.I. virus for embryonating eggs was essentially the same by various routes of inoculation. Comparable distribution and concentrations of virus were obtainable following allantoic chamber and intravenous inoculation. Eggs incubated ten to twelve days prior to inoculation were most satisfactory for use in the preparation of vaccines.

4) The virulence of the viruses for chickens and chicken embryos and/or their immunogenic properties were highly stable under both optimal and certain suboptimal conditions of propagation in serial groups of embryonating eggs.

5) Formalin and ultraviolet light of 1,600 to 1,800 Å wave length were highly useful for the destruction of virus infectivity in the preparation of inactivated virus vaccines; the former possessed the advantages of greater simplicity and economy. Irradiation with ultraviolet light of longer wave lengths (2537 Å) resulted in the loss of both infectivity and immunogenicity.

6) Variations in age ranging from 1 week to 4 years were without effect on the immunity response to vaccination. Likewise, neither the breed or sex of chickens nor the treatment of cockerels with stilbesterol demonstrably influenced the vaccinal response.

7) The unsatisfactorily irregular and low immunity responses to inactivated virus in embryo components was favorably affected by the addition of certain oily materials and of alumina gel. While not significantly unlike that of alumina gel, the adjuvant effect of Falba and mineral oil, in concentrations of 5 to 10 per cent and of 20 to 25 per cent, respectively, was highly beneficial, according to extensive studies. Larger amounts of these oily adjuvants, the substitution of peanut oil for the mineral oil, or the addition of killed *Mycobacterium butyricum* resulted either in no significant increase in the immune responses or in the production of objectionable changes in the tissues.

8) Maximal immunity resulted from vaccination with nonadjuvant vaccines followed after several months by exposure to the D.E.I. virus. The degree of this immunity was virtually equalled by injection of the living variant viruses. Lesser measures of response were usually evoked by the inactivated virus vaccines in the following descending order: adjuvant vaccines, divided doses of nonadjuvant vaccines and, finally, single doses of the plain embryo-component vaccines.

9) The immunogenicity of formalinized virus in nonadjuvant vaccines rapidly de-

teriorated at a pH level that was destructive to its viability, or at pH 5. Within the range of pH 6 to 9, the vaccine was apparently unaltered for a period of 142 days. Variable evidences of immunogenicity, although sufficient to protect 50 per cent or more of the chickens, were retained for as long as 611 days.

10) The living variant viruses were capable of provoking a high grade immunity when injected into susceptible chickens and when introduced onto the surfaces of the nasal passages, cloaca, and feather follicles. The dosage, within a wide range, was of little or no consequence on the resultant immunity. Apparent differences in the immunizing properties were demonstrated between two variant strains.

11) The appearance of refractivity to challenge exposure to the D.E.I. virus occurred within two to four days following injection of living variant virus. Corresponding evidences of immune responses to inactivated virus were shown to occur two to four days later, or between four to eight days postvaccination. Significant titers of infectivity-neutralizing humoral antibodies were demonstrable at ten and twelve days, respectively, after vaccination with the living and the inactivated virus vaccines.

12) An immunity of a high degree persisted for as long as eighteen to twenty-one weeks, the longest periods studied, following vaccination with whole egg adjuvant vaccines and with living variant virus. The postchallenge residual reactions in the tissues indicated, however, that these chickens were not solidly immune to severe challenge exposure to the virus. Nevertheless, the highly practical value of the immunity was shown by the prevention of clinical evidences of illness.

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